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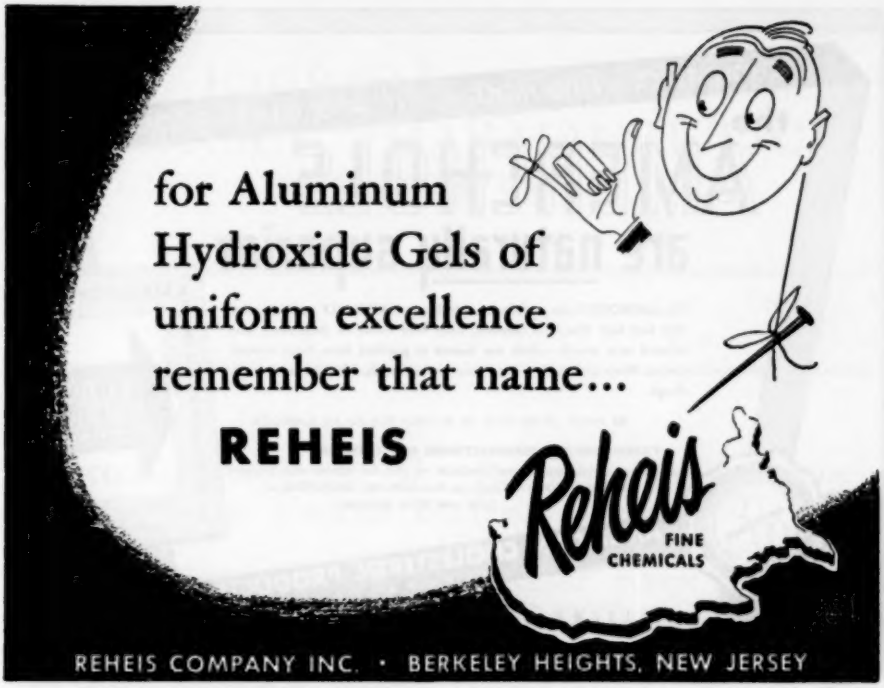

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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

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Bacterial Pyrogens. II. Pyrogenic Preparations from Various Bacterial Species*,†

By LEONARD G. GINGER, N. M. NESSET, BYRON RIEGEL, and EDWARD J. FITZSIMONS

Essential differences in the biological activity and chemical composition of pyrogenic concentrates prepared from five species of bacteria are shown. Particular attention is devoted to the significance of the total pyrogenic polysaccharide content of each concentrate, as well as to the amount of reducing sugar, hexosamine and nonreducing carbohydrate which it contains.

PYROGENIC CONCENTRATES have been prepared from *Escherichia coli*, *Salmonella typhi*, *Bacillus subtilis*, *Serratia marcescens*, and *Proteus vulgaris* by a uniform method similar to that described for a *Pseudomonas* species (1). A detailed comparison of the chemical composition and biological activity of these pyrogenic concentrates has been made.

Pyrogenic preparations from these bacterial species have been described by other investigators (2–21). The work in this field has been reviewed by Co Tui (22) and by Bennett and Beeson (23). A direct comparison of the chemical and biological properties of such preparations is not practical because the conditions for growth of the organisms and the techniques employed in isolating the active materials were not uniform. The essential question of whether pyrogens from various bacterial species are the same or different has not been answered.

The method of tryptic digestion, which was employed in this study in obtaining pyrogenic concentrates, did not yield preparations free from all extraneous materials; however, it did provide similarly prepared materials for comparison. The evidence indicates that there are differences in the biological activity of the five concentrates included in this study which are probably related to inherent differences in the composition of the pyrogenic polysaccharides.

EXPERIMENTAL[1]

Cultivation of Organisms.—The following bacteria from the American Type Culture Collection were used in this investigation: *E. coli* (A.T.C.C. No. 8739), *S. typhi* (A.T.C.C. No. 6539), *B. subtilis* (A.T.C.C. No. 6633), *S. marcescens* (A.T.C.C. No. 9986), and *Pr. vulgaris* (A.T.C.C. No. 6380). The cells were grown and collected in the manner described previously for a *Pseudomonas* species (1), except that inocula of 200 ml. of twenty-four-hour cultures were added to 15 L. of broth. Four of the bacterial species were grown on Difco nutrient broth. *Pr. vulgaris*, however, was grown on a broth containing 5% protein hydrolysate and 0.5% glucose.

* Received May 3, 1951, from the Research Laboratories, Baxter Laboratories, Inc., Morton Grove, Ill., and the Chemical Laboratory, Northwestern University, Evanston, Ill.

† The present report is part of a cooperative investigation of pyrogens. The work at Northwestern University has been supported by funds provided by the Baxter Laboratories.

[1] The authors wish to acknowledge the technical assistance of Paul Z. Anthony, Walter Kuhn, Richard Lamey, James McLallen, and Mary M. Neal.

TABLE I.—QUANTITATIVE COMPOSITION STUDY

Component	*S. typhi* Cells, %	*S. typhi* Concentrate, %	*Pr. vulgaris* Cells, %	*Pr. vulgaris* Concentrate, %	*E. coli* Cells, %	*E. coli* Concentrate, %	*S. marcescens* Cells, %	*S. marcescens* Concentrate, %	*B. subtilis* Cells, %	*B. subtilis* Concentrate, %
DNA	1.9	2.8	4.7	10.1	4.5	10.5	4.6	11.5	1.1	1.5
RNA	4.8	6.0	2.9	4.4	5.4	6.5	8.0	12.2	2.0	3.8
Hexosamine (A)	1.1	1.6	2.8	4.1	1.8	3.0	1.1	2.7	7.1	11.0
Total nitrogen	12.0	6.7	12.8	8.0	13.4	7.6	12.6	7.0	6.2	4.4
Protein	67.7	...	71.1	...	72.7	...	65.3	...	32.2	...
Nitrogenous residue	...	32.2	...	32.9	...	28.4	...	18.0	...	16.7
Free lipid	3.5	2.1	3.7	0.7	2.7	1.3	2.6	1.9	3.0	0.9
Ash	4.4	10.3	2.9	16.1	5.4	11.8	7.9	10.4	16.5	13.9
Total known components	83.4	55.0	88.1	68.3	92.5	61.5	89.5	56.7	61.9	47.8
Unclassified polysaccharide (B)	16.6	45.0	11.9	31.7	7.5	38.5	10.5	43.3	38.1	52.2
Pyrogenic polysaccharide (A + B)	17.7	46.6	14.7	35.8	9.3	41.5	11.6	46.0	45.2	63.2

Preparation of Concentrates.—The various cellular materials were subjected to digestion with trypsin to degrade the cellular proteins (1). Concentrates were obtained in 25–35% yields from *E. coli*, *S. marcescens*, and *Pr. vulgaris* cells, while *S. typhi* and *B. subtilis* cells gave 45–55% yields of concentrate.

Composition.—The cellular materials and their corresponding concentrates were analyzed for desoxyribonucleic acid (DNA), ribonucleic acid (RNA), hexosamine, total nitrogen, protein or nitrogenous residues, free lipid, ash, and pyrogenic polysaccharides, using previously described methods (1). Comparative results for representative cellular materials and their corresponding concentrates are presented in Table I.

The nitrogen in the cellular materials attributable to DNA, RNA, and hexosamine was computed and summed. It was assumed that the nitrogen in the cells, not assignable to these three nitrogenous substances, represents protein nitrogen. Computation gave the values shown in Table I for the protein content of the cells.

The DNA, RNA, and hexosamine do not account for all of the nitrogen in the concentrates. The presence of nitrogenous residues, at least partially of a peptide nature, is indicated. Positive biuret reactions have confirmed the presence of such peptide linkages. The amount of these nitrogenous residues in the concentrates (Table I) was computed from the nitrogen not attributable to DNA, RNA, or hexosamine.

The known components for the cells and concentrates were totaled, excluding total nitrogen, since this is already included in the nitrogenous components (Table I). The difference between each sum obtained and 100% represents unclassified polysaccharide. The hexosamine which is liberated upon hydrolysis is probably an essential unit in the pyrogenic polysaccharide. Therefore, its value has been added to the unclassified polysaccharide to give a value representative of the total amount of pyrogenic polysaccharide present.

The total reducing sugar content (expressed as glucose) of the various concentrates was determined by the method of Shaffer and Hartman (24). The summed contribution of the sugars in the nucleic acids and the hexosamine to the total reducing sugar (all expressed as glucose) was calculated. These values and other characterization data concerning the carbohydrates in the concentrates are presented in Table II. The difference between the value for unclassified reducing sugar and the value representing unclassified polysaccharide gives a value representative of the nonreducing carbohydrate present.

Biological Activity.—The minimum pyrogenic dose (MPD), a concept introduced by Co Tui, *et al.* (14), was used in comparing the biological activities of the various concentrates. The maximal febrile responses which they elicited were determined following intravenous administration into rabbits by a method described previously (1). At least 12, and in some cases as many as 30, rabbits were employed at each of a number of dose levels. The temperatures were measured with rectal resistance bulbs and recorded automatically, using a 15-point Foxboro Rabbit Scanner and Fever Recorder.[2] The average maximal temperature re-

[2] Measurements were made by Dr. M. E. Pinkerton o Baxter Laboratories, Inc.

TABLE II.—CHARACTERIZATION OF CARBOHYDRATES IN CONCENTRATES

Carbohydrate	*S. typhi*, %	*Pr. vulgaris*, %	*E. coli*, %	*S. marcescens*, %	*B. subtilis*, %
DNA pentose[a]	0.4	1.6	1.7	1.8	0.3
RNA pentose[a]	1.9	1.4	2.1	3.8	1.3
Hexosamine[a]	1.5	4.1	3.0	2.7	11.0
Total classified	3.8	7.1	6.8	8.3	12.6
Total reducing sugar[a]	5.1	9.6	9.3	11.9	23.9
Unclassified reducing sugar (C)	1.3	2.5	2.5	3.6	11.3
Unclassified polysaccharide (B)	45.0	31.7	38.5	43.3	52.2
Nonreducing carbohydrate (B-C)	43.7	29.2	36.0	39.7	40.9

[a] Results expressed as glucose.

sponse for each concentrate at each dose level studied is shown in Fig. 1. The dosage corresponding to the point of intersection of the fever curve for each concentrate and the line representing a febrile response of 1° F. represents the MPD. It is apparent that *S. typhi* concentrate is the most active preparation, having a MPD of 0.22 γ/Kg. Almost as active are *Pr. vulgaris* concentrate with a MPD of 0.32 γ/Kg. and *E. coli* concentrate with a MPD of 0.40 γ/Kg. *S. marcescens* concentrate has a MPD of 0.75 γ/Kg., and *B. subtilis* concentrate is much weaker than the other four with a MPD of 7.5 γ/Kg.

DISCUSSION

The preparation of pyrogenic concentrates from five bacterial species, by subjecting the cellular materials to tryptic digestion, has yielded information that indicates that there are essential differences in the chemical composition and biological activity of these concentrates. It is readily apparent (Table I and Fig. 1) that chemically and biologically the concentrate prepared from *B. subtilis* cells is distinctly different from the concentrates prepared from the other four cellular species, which differ from each other to a lesser degree.

At least three possible contaminants are associated with the pyrogenic polysaccharides, namely, nucleic acids, other nitrogenous material (at least partially of a peptide nature), and free lipid (Table I), the latter being present in moderately small quantity.

The amount of contaminating nucleic acids in each concentrate is related to the initial nucleic acid content of the cellular material. Considering the yield of each concentrate obtained, very little of the DNA is removed in preparing the concentrates, whereas this is not so for RNA (Table I).

The amount of nitrogenous residues present in the concentrates varies with the cellular species under consideration. Since the initial protein contents of the various cellular materials are similar, it appears that the proteins are not degraded to the same extent by tryptic digestion, so that the amount of nondialyzable peptides remaining as a contaminant varies. On the other hand, the possibility of the presence of a polysaccharide-amino-acid complex in the concentrates must not be overlooked. This would not be a unique finding, since Aminoff, Morgan, and Watkins (25), for example, have reported that the human blood group A substance is an essentially homogeneous mucoid or polysaccharide-amino-acid complex on the basis of physical, chemical, and immunological measurements. Variations in the amounts of the nitrogenous residues (Table I) contained in the concentrates from the various bacterial species could be due, at least partially, to the complexing of different amounts of amino acids by each polysaccharide moiety.

By making several assumptions, it has been possible to compute the amount of pyrogenic polysaccharide present in each of the concentrates studied (Table I). *Pr. vulgaris* concentrate was found to contain the least amount of pyrogenic polysaccharide (35.8%) and *B. subtilis* concentrate to contain the most (63.2%); however, the former is more active biologically. The pyrogenic polysaccharides have been shown to consist of hexosamine, an unclassified reducing sugar, and a nonreducing fraction. These components must be considered in detail in attempting to correlate pyrogenicity with quantitative chemical composition. The hexosamine and the unclassified reducing sugar are present in approximately a 1:1 ratio in each of the concentrates; however, there seems to be no direct correlation between the pyrogenicity of the four more active concentrates and the hexosamine or the unclassified reducing sugar contents. *S. typhi* concentrate, which is the most active, has the smallest amount of these two components (Table II). This lack of direct

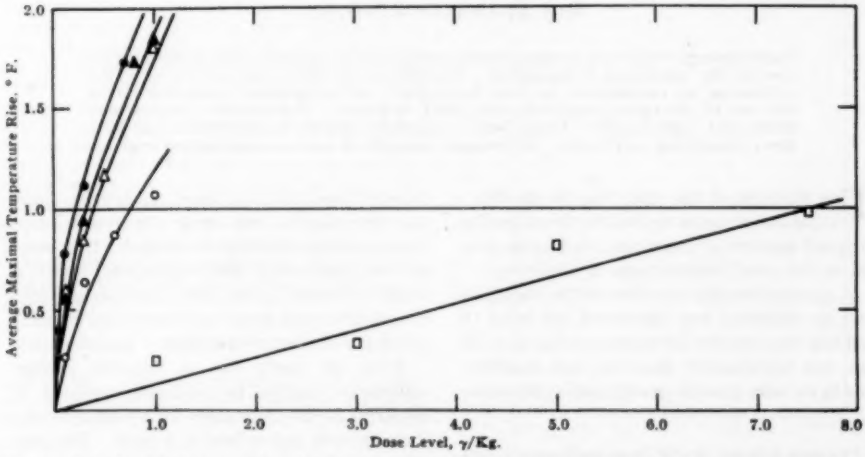

Fig. 1.—Relationship between dose level of concentrate and average maximal febrile response in rabbits. ●, *S. typhi*; ▲, *Pr. vulgaris*; Δ, *E. coli*; ○, *S. marcescens*; □, *B. subtilis*.

correlation is especially apparent when *B. subtilis* concentrate is considered. It has the largest amount of hexosamine (11.1%) and unclassified reducing sugar (11.3%) of the preparations studied, but it is the weakest biologically. There does not seem to be a significant correlation between biological activity and nonreducing carbohydrate content, although the most active concentrate, that from *S. typhi*, does have the highest nonreducing carbohydrate content (43.7%).

CONCLUSIONS

The available evidence indicates that there are essential differences in the biological activity and chemical composition of the five pyrogenic polysaccharides investigated and that it is impossible to correlate directly pyrogenicity with existing composition data. It is possible that some yet undetermined component is responsible for the observed differences in biological activity. Similarly, the nature of the unclassified reducing sugar, or the nonreducing carbohydrate, may be important. Finally, the possibility that biological activity may be related to differences in molecular structure cannot be overlooked. Further studies are planned to evaluate these hypotheses.

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A Colorimetric Method for the Estimation of Colchicine*

By J. STANTON KING, Jr.†

A colorimetric technique for the general quantitative adaptation of the Zeisel qualitative test for colchicine is described. The method involves the conversion of the colchicine to colchiceine by acid hydrolysis and subsequent measurement at 470 mμ of the color produced when $FeCl_3$ is added. The method is rapid, sensitive, and reproducible. It has been successfully applied to pharmaceutical mixtures containing colchicine. A detailed example of such an application is given.

THE PURPOSE of this study was to develop a rapid and accurate method for the estimation of small amounts of colchicine, such as are present in the usual preparations of this drug.

A qualitative color reaction for the identification of colchicine was discovered by Zeisel (1) and has been applied to specific problems (2, 3), but was inadequately described and characterized in its more general quantitative applications. It was found that the Zeisel reaction is simple and reproducible and meets the requirements for a practical colorimetric method. It is based on the observation that colchiceine, which is readily obtained by the acid hydrolysis of colchicine, forms a jade-green color with ferric chloride, which is suitable for quantitative measurement.

While this study was in progress, another colorimetric method for colchicine appeared (4) involving a color formation with hydroxylamine hydrochloride and sodium hydroxide. The present method is equally as simple and about ten times as sensitive.

* Received February 19, 1951, from the Research Laboratory of The S. E. Massengill Co., Bristol, Tenn.
† The author is indebted to Dr. Georg Cronheim for his helpful supervision of this study.

EXPERIMENTAL

Reagents.—(*a*) Ferric chloride, C. P., 5% solution in 1.0 *N* HCl. This need not be a freshly prepared solution.

(*b*) Colchicine. For purification, a chloroform solution of the U. S. P. alkaloid was treated with Norit A, filtered, an equal volume of ethyl ether added, and the alkaloid reprecipitated with petroleum benzin. It melted at 153–155° (uncorr.) after drying for three hours at 102°.

(*c*) HCl, 1.0 *N*.

(*d*) Chloroform, U. S. P.

PROCEDURE

Pure colchicine alkaloid (2.5 mg.) is dissolved in 25 ml. of 1.0 *N* HCl in a 125-ml. Erlenmeyer flask. A funnel is inserted into the neck of the flask, which is then warmed on the steam bath for one hour. The flask is removed, cooled to room temperature, and the volume made to exactly 25 ml. with 1.0 *N* HCl. Graduated amounts containing up to 0.5 mg. of this solution are made to 5 ml. with 1.0 *N* HCl, and 0.1 ml. of a 5% solution of $FeCl_3$ is mixed with each. These solutions are then read in a colorimeter set for maximum transmittance at 470 mμ. This establishes the standard curve, which follows Beer's law up to 0.5 mg. (Fig. 1).[1] Samples of unknown colchicine content are run in the same way, after separation of interfering materials.

The following procedure constitutes a specific example of an application of this method to a pharmaceutical product. The preparation was an ampul which contained in 20 ml.:

2.0 Gm. sodium salicylate
2.0 Gm. sodium iodide
0.32 Gm. sodium cacodylate
0.65 mg. colchicine alkaloid

One normal HCl is added to 20 ml. of the preparation until the salicylic acid just begins to precipitate (about *p*H 4.2). Water is added to make a volume of 40 ml. and the mixture vigorously extracted with three 30-ml. portions of chloroform. The combined chloroform extracts are washed with one 15- and two 10-ml. portions of water to remove iodide and cacodylate, and then evaporated to dryness on the steam bath in a 125-ml. Erlenmeyer flask. Eighteen milliliters of 1.0 *N* HCl are added and the procedure completed as for the standard, except that, after hydrolysis is complete, the flask is allowed to stand in ice water until all excess salicylic acid has precipitated, and the mixture then filtered. The filtrate is brought to exactly 20 ml. by successive washings of the filter with 1.0 *N* HCl.

At this *p*H and this concentration, the violet iron-salicylate color has an optical density at 470 mμ of only about 0.04.

RESULTS

The color is formed immediately. Its intensity makes it suitable for the identification of as little as 4 μg. of colchicine on a spot plate. Figure 2 gives

[1] In all this study, except Fig. 3, the Beckman DU Spectrophotometer was used. In Fig. 3 the Coleman Model 11 Spectrophotometer was used.

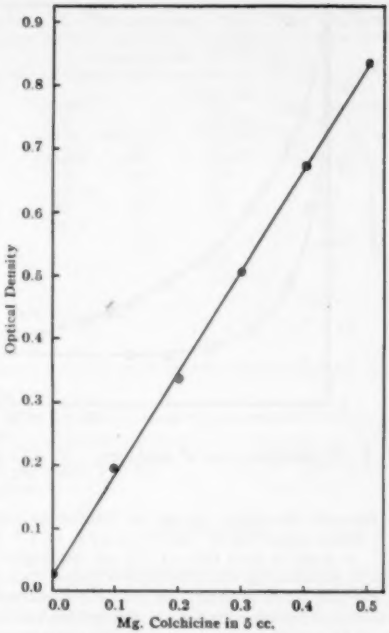

Fig. 1.—Standard curve for colchicine.

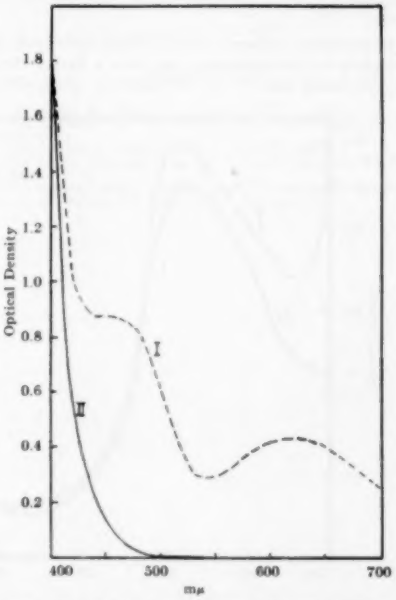

Fig. 2.—Spectral absorption curve for 10 mg. % colchicine (as iron-colchiceine) (I) and $FeCl_3$ alone (II)

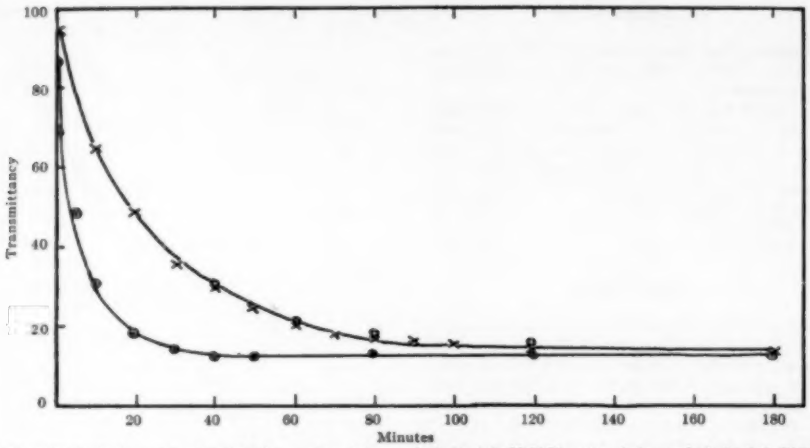

Fig. 3.—Hydrolysis rates of colchicine. ⊙—0.1 mg./ml. in 1.0 *N* HCl; ×—0.5 mg./ml. in 0.1 *N* HCl; ○—0.1 mg./ml. in 0.1 *N* HCl.

the spectral absorption curves for colchiceine and for a blank consisting of the $FeCl_3$ only, in 1.0 *N* HCl. It may be seen that at 470 mμ the highest value is obtained for the difference between sample reading and blank reading, and that the maximum at 630 mμ is considerably lower. Several such readings at 470 mμ gave an average specific absorbancy ($E_{1\,cm.}^{1\%}$) of 85, after subtracting the reading given by the $FeCl_3$ alone.

Ferric nitrate is less suitable than ferric chloride, because it gives a yellowish-tinged and less intense green.

Experiments showed (Fig. 3) that hydrolysis is complete in forty minutes, but that a longer time on the steam bath is not detrimental. Hydrolysis time is also independent of alkaloidal concentration, up to at least 0.5 mg. colchicine per ml. Neither the acidity nor the $FeCl_3$ concentration have to be controlled with great care. The green color has good light-, time-, and acid-stability. These facts facilitate easy reproducibility. A colchiceine solution of 0.1 mg./ml. in 1 *N* HCl gave the same reading after seven weeks when ferric chloride was added.

To gain a better conception of the sensitivity of the method, solutions containing from 10 to 100 μg. of colchicine, as colchiceine, in 3.0 ml. of 1.0 *N* HCl had 0.1 ml. of 5% $FeCl_3$ added and the resultant color read. The results (Table I) indicate the precision of the method on a micro scale to be approximately ±1 μg.

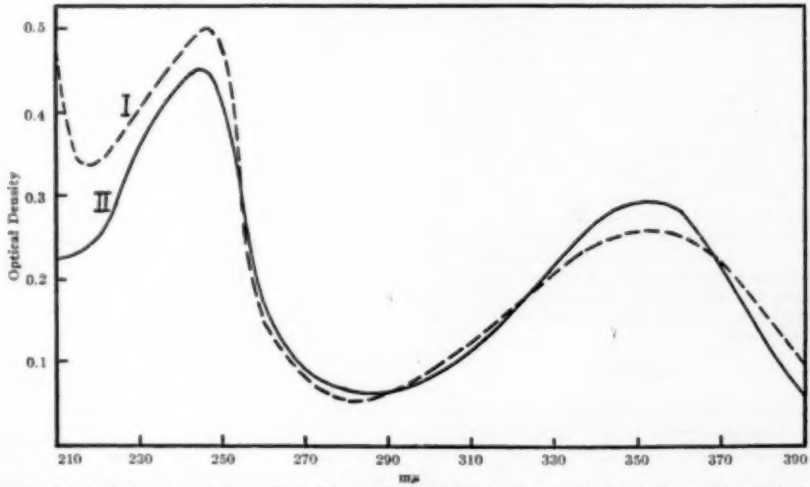

Fig. 4.—Spectral absorption curves for 0.75 mg. % colchicine in water (II) and 0.75 mg. % colchiceine in 1.0 *N* HCl (I).

TABLE I.—OPTICAL DENSITY READINGS AT 470 mμ FOR COLCHICINE (AS IRON-COLCHICEINE)

μg. Colchicine in 3 Ml.	Optical Density Reading
0	0.038
20	0.097
40	0.159
60	0.215
80	0.270
100	0.335

Interference in the present method could reasonably be expected from any phenols or enols capable of reacting like colchicine with $FeCl_3$, or from other $CHCl_3$-extractable, acid-stable substances absorbing light at 470 mμ. However, in several of the usual colchicine preparations tested, no such interference was encountered which could not be overcome by the procedure described or some simple modification of it.

Before hydrolysis is begun, an aliquot of the isolated alkaloid should be tested for colchiceine (5) to be sure none is initially present as an impurity.

Extraction of colchicine from weakly alkaline solutions by chloroform is poor (Table II), but recovery is quantitative from acid solutions. In the presence of salicylate, a common ingredient in pharmaceutical preparations containing colchicine, there is an apparent recovery of only 70–80% with either three or six 30-ml. chloroform extractions. Although the reason for this is not presently clear, the recovery can be made quantitative by adding sodium chloride or sodium iodide to the mixture. These salts do not contribute any color in the procedure.

Typical results (in per cent of labeled amount) of assays of ampul preparations of the sort given in the procedure above were: 98, 97, 100, 95, and 107. These figures represent a variety of batches and manufacturers.

TABLE II.—RECOVERIES IN % OF 2 MG. COLCHICINE FROM 40 ML. AQUEOUS VOLUME BY THREE 30-ML. CHLOROFORM EXTRACTIONS

Colchicine Alone Weakly Alkaline Soln.	Colchicine Alone Acid Soln.	Colchicine with Sodium Salicylate (10%), Acid Soln.	Colchicine with Sodium Salicylate (10%) and Sodium Chloride (10%) Acid Soln.
72	100	76	100
77	100	76	104
78	98	70	95
..	98	75	96
..	..	78	98
..	..	74[a]	..

[a] Six extractions.

SUMMARY

A colorimetric technique for the general quantitative adaptation of the Zeisel test for colchicine is described. This method is rapid and accurate, and is suitable for microanalytical application.

The procedure has been successfully applied to pharmaceutical mixtures containing colchicine. A detailed example of such an application is given.

ADDENDUM

While direct measurement of colchicine absorption in the ultraviolet region is more sensitive, it is too susceptible to interference from other ingredients in a mixture. The ultraviolet absorption curve is nearly the same for colchicine and for colchiceine. Using a hydrolyzed and undrolyzed 0.75 mg. per cent solution of colchicine, curves (Fig. 4) were obtained for colchicine in water and colchiceine in 1.0 *N* HCl.

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Phagocyticidal and Antibacterial Action of Boric Acid*,†

By MILAN NOVAK and WELTON I. TAYLOR

Experiments *in vitro* at temperatures of 37° or 40° indicate that boric acid in concentrations higher than 2 per cent inhibits phagocytosis. No inhibition is demonstrated by boric acid in concentrations below 2 per cent at these same temperatures. Several suggestions and recommendations relative to the clinical use of boric acid and based on these findings are made.

BORIC ACID has become a commonly employed antiseptic agent in certain clinical conditions through the medium of clinical evaluation alone. Its use was introduced by Godlee in 1873 as a companion drug to Lister's use of carbolic acid (1). Perhaps this association in a prebacteriological era explains its wide acceptance in clinical use, as well as the lack of any significant number of accurate laboratory studies on its comparative value as a germicide and as a topically toxic agent to tissue cells.

Recent reports on the systemic toxicity of boric acid when taken accidently by the oral route and the introduction of newer antibacterial agents including sulfonamides, antibiotics, and quaternary compounds has re-emphasized a need for the scientific evaluation of its tissue toxicity and action as a germicide. To this end we have directed our laboratory studies and have arrived at some practical suggestions and conclusions.

MATERIALS AND METHODS

Blood was drawn aseptically from healthy adult male donors in approximately 15-ml. amounts and shaken for fifteen minutes in sterile 50-ml. Erlenmeyer flasks containing 30 to 50 glass beads to defibrinate. Sterile (supersaturated where necessary) boric acid solutions in saline were added to chemically clean 10 x 75-mm. test tubes in 0.5-ml. amounts. The addition of 0.5-ml. amounts of freshly defibrinated whole blood to these tubes then yielded boric acid concentrations of 4.8, 3.6, 2.4, 1.2, 0.6, 0.3, 0.15%, and a control set having sterile saline instead of boric acid. The tubes were corked tightly with size 1 corks which had been soaked in distilled water for seventeen days with daily or twice daily changes of water to remove acids which interfere with phagocytosis. These tubes in duplicate were then placed on a metal disk 8 in. in diameter which had channels soldered on to act as racks for the test tubes. The disk with the affixed tubes was then put on the drive shaft of a $^1/_{60}$-hp. electric motor with a 288 : 1 reduction gear ratio fastened to a ringstand with a C clamp so that the extended drive shaft entered the 26-L. water bath at approximately a 40° angle and the disk and tubes were immersed. A switch box with rheostat completed the equipment and allowed the disk to be rotated at 12 r. p. m. to secure adequate mixing of the blood and boric acid solutions. This method is adapted from that of Welch and Hunter (2), as modified and illustrated by Hanson (3).

The tubes were rotated in a constant temperature (±1°) water bath for thirty minutes, at which time they were removed and 0.2 ml. of a suspension of *Micrococcus pyogenes* var. *albus*[1] which was easily phagocytized was added. The concentrations then became 4, 3, 2, 1, 0.5, 0.25, 0.125, and 0% boric acid. The time required for this step was about twenty minutes. The tubes were replaced, immersed, and rotated in the water bath for an additional fifteen minutes. Upon removal, phagocytosis was halted by running cold tap water over the tubes while agitating them for five minutes.

The cocci used were grown on nutrient agar slants for forty-eight hours, washed off with sterile distilled water, adjusted to the density of a $BaSO_4$ nephelometer tube No. 1 with a Coleman Junior Spectrophotometer, and kept refrigerated at 4° when not in use. The same suspension was used for all experiments over the period of a month.

After chilling the tubes, a drop of the blood mixture was placed on the end of a clean microslide with a Wright's pipette, and routine thick smears were made. Wright's stain was used. One hundred polymorphonuclear neutrophils were observed on each slide and the degree of phagocytosis was noted according to the following scale: 0–5 cocci ingested by the PMN was considered negative (since cocci on or below the cell seem to be within); 6–20 cocci, slight; 21–40 cocci, moderate; 41 or more cocci, maximal phagocytosis.

The experiments were performed at three temperatures, 34, 37, and 40°, to correspond roughly to the temperature of the skin, normal body temperature, and in febrile patients, respectively. Blood from three different donors was used at each temperature and at all concentrations. Each such experiment was carried out in duplicate.

Some indication of the effect of boric acid concentrations on the organism used in the phagocytosis experiments was desirable, so the following experiment was performed.

Wassermann tubes containing 2 ml. of double strength brain-heart infusion broth and 2 ml. of sterile boric acid in saline of such strength (supersaturated where necessary) as to make final con-

* Received March 28, 1951, from the Department of Bacteriology, University of Illinois, College of Medicine.
† Prepared under the auspices of the Therapeutic Research Foundation, 810 Eighteenth Street, N. W., Washington 6, D. C.

[1] Originally isolated from skin of a patient.

centrations comparable to those in phagocytosis experiments received 0.8 ml. of a twenty-one-hour brain-heart infusion broth culture of *M. pyogenes* var. *albus*. The tubes, which had been warmed previously to 37°, were incubated for thirty minutes at 37°. Duplicate sets of brain-heart infusion agar plates were poured using 1 ml. of the cultures. Plates were incubated at 37° and read at forty-eight hours. Results are shown in Table I.

TABLE I.—EFFECT OF A THIRTY-MINUTE EXPOSURE OF M. PYOGENES VAR. ALBUS TO BORIC ACID SOLUTIONS

Boric Acid, %	Plate Counts		Av. Counts
	Set 1	Set 2	
0.0	35	24	30
0.125	28	23	26
0.25	21	27	24
0.5	42	33	38
1.0	15	27	21
2.0	19	20	20
3.0	9	9	9
4.0	5	2	4

RESULTS

Results of the averages of 600 phagocytes from three donors in duplicate at each of the temperatures at which experiments were performed are shown in Fig. 1.

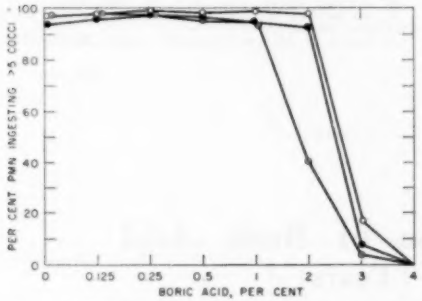

Fig. 1.—Effect of temperature on phagocytosis. ○, 37°, ● 40°, ⊙, 34°.

The degree of phagocytosis with respect to relative numbers of cocci ingested at 37° is represented by Fig. 2. Phagocytosis at 40° is illustrated by Fig. 3. Phagocytosis at 34° is shown in Fig. 4. Each of these represents averages of 600 phagocytes per boric acid concentration from three donors, except as footnoted. The greatest number of cocci were ingested at 40°, somewhat fewer at 37°, and considerably fewer at 34°.

DISCUSSION

The action of phagocytes is not inhibited or stimulated to any great degree by concentrations of boric acid up to and including 2% at 37 or 40° (Figs. 1–3). At 34°, however, there is a marked decline in phagocytosis at 2% boric acid concentration (Figs. 1 and 4). A concentration of 3% boric acid results in a

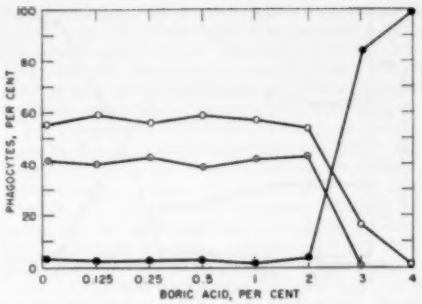

Fig. 2.—Quantitative evaluation of phagocytosis at 37°. ●, 0–5 cocci/pmn. ○, 6–20 cocci/pmn. ⊙, 21–40 cocci/pmn.

considerable decrease in phagocytosis at all temperatures of the experiment, and the loss of power to ingest more than five cocci is complete at 4% boric acid concentration.

Smears stained with Wright's stain show a change in coloration in the polymorphonuclear neutrophils and a more heavily stained granular appearance of these cells which is similar to the appearance of toxic granulation at boric acid concentrations of 3 and 4%.

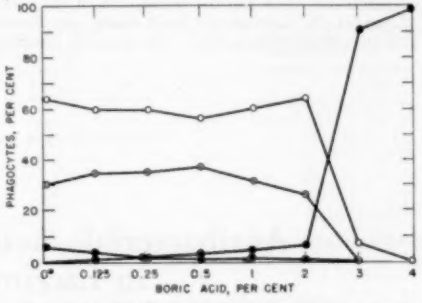

Fig. 3.—Quantitative evaluation of phagocytosis at 40°. ●, 0–5 cocci/pmn. ○, 6–20 cocci/pmn. ⊙, 21–40 cocci/pmn. ⊗, >40 cocci/pmn. Asterisk denotes two donors only.

Boric acid is soluble at room temperature to the extent of approximately 5%, and this concentration is often used clinically as a so-called mild antiseptic. It is to be considered, therefore, that concentrations greater than 2% may inhibit phagocytosis, thereby negating a primary defense mechanism of the body against bacterial invasion. The solubility of boric acid at 0° is 1.95%, and use of a solution kept at refrigerator temperature (approximately 4°) or prepared by dissolving boric acid crystals in water at refrigerator temperature would thus prevent phagocyticidal toxicity.

Results of the *in vitro* testing of the micrococcus used throughout these experiments (Table I) show that 4% boric acid was not toxic to 100% of the cocci, but was toxic to 100% of the phagocytes under the conditions previously described and shown in

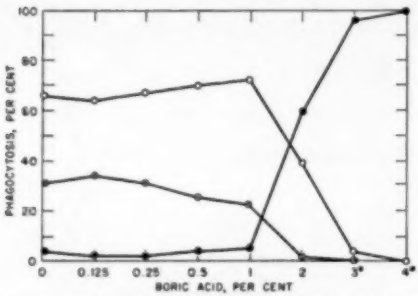

Fig. 4.—Quantitative evaluation of phagocytosis at 34°. ●, 0–5 cocci/pmn. ○, 6–20 cocci/pmn. ⊙, 21–40 cocci/pmn. Asterisk denotes two donors only.

Figs. 1 and 2. Admitting the differences in techniques employed, comparison of these data to formulate a toxicity index would give a number greater than one, indicating the tissue toxicity to be greater than the bactericidal activity of the boric acid at 4% concentrations [Salle, *et al.* (4)]. An appreciable number of organisms, however, are killed in 2% boric acid (nontoxic concentration) for the 37° and 40° temperatures. It has been repeatedly demonstrated that bacterial cells of many pathogenic species are less susceptible to the lethal action of germicides than are the tissue cells of the body [Lambert (5), German (6), Buchsbaum and Bloom (7), Hirsch and Novak (8)]. The invasive qualities of these organisms may thus be aided and abetted by the injudicious use of too concentrated a solution of boric acid.

SUMMARY

Phagocytosis is inhibited by concentrations of boric acid higher than 2 per cent at temperatures of 37° or 40° under the conditions of the *in vitro* experiments performed.

Phagocytosis is partially inhibited by 2 per cent boric acid concentration, and totally inhibited by higher concentrations at 34°.

The need for an increased awareness of these data in the clinical use of boric acid is discussed. It is recommended that refrigerated boric acid solutions be prepared to insure the clinical use of non-phagocyticidal concentrations.

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Antibacterial Action of Boric Acid in Lacrima (Tears)*,†

By MILAN NOVAK and WELTON I. TAYLOR

Boric acid in concentrations of from 0.5 to 2 per cent exerted bacteriostatic action on broth cultures of typical pyogenic organisms often found in the eye. This action was not inhibited by lacrimal secretions. Results indicate that tears may increase slightly the bacteriostatic action of boric acid.

THE common use of boric acid solutions for application to the eye in cases of injury or infection raises the question as to the influence of lacrimal secretions on the bactericidal or bacteriostatic action of this germicide. Since boric acid is generally regarded as a mild germicidal agent, any reduction of its antibacterial action by such environmental factors as tears might limit its rational use in pathological conditions involving the eye. The present study was initiated to determine its action in the presence of tears on representative organisms often found in minor eye infections.

MATERIALS AND METHODS

Three species of bacteria were selected to test the inhibitory effect of various concentrations of boric acid under ordinary conditions of laboratory testing. *Micrococcus pyogenes* var. *albus*, originally isolated from normal skin, a *beta*-hemolytic streptococcus and *Diplococcus pneumoniae* were grown in

* Received March 28, 1951, from the Department of Bacteriology, University of Illinois, College of Medicine.
† This investigation was conducted under the auspices of the Therapeutic Research Foundation, 810 Eighteenth Street, N. W., Washington 6, D. C.

brain heart infusion broth for twenty-four hours. Wassermann tubes containing 2 ml. of double strength brain-heart infusion broth and 2 ml. of sterile boric acid in saline in such concentrations (supersaturated where necessary) to obtain final concentrations of 4, 3, 2, 1, 0.5, and 0.25% and sterile saline for control series, received 0.8 ml. of a 10^{-6} dilution of a twenty-four hour culture. The tubes were incubated at 37° and 1 ml. of inocula removed at three, six, and twenty-four hours and plated in brain-heart infusion agar. Plates were incubated at 37° for forty-eight hours. Resulting counts are shown in Table I.

Results shown in Table I illustrate the mild action of boric acid against the bacterial species used. All concentrations of boric acid inhibited reproduction of all bacterial species tested as compared with controls. A diminution of colonies at twenty-four hours over colonies at three hours was obtained at boric acid concentrations of 1% for *M. pyogenes* var. *albus*, 2% for the streptococcus, and 0.5% for *D. pneumoniae*. These concentrations can be interpreted as bacteriostatic levels and are comparable to a number of previously reported studies.

Bactericidal end points are reached at boric acid concentrations above those just inhibiting growth, as is demonstrated by a marked reduction in colony count in the higher concentrations of boric acid. The frequent absence of complete killing may be explained by the presence of highly resistant individual cells which have been observed with numerous antibacterial agents. The data, however, indicate that the three species used in these studies were of average resistance and are therefore representative organisms for further study with tears.

An investigation of the effect of boric acid on bacteria in the presence of tears was then conducted. Tears were obtained from healthy adult subjects using cigarette smoke as a lacrimator. As tears formed they were drawn into sterile Wright's pipettes by the combined means of capillary action and gravitational flow. Tears collected from seven subjects were pooled, a portion used immediately (Table II) and the remainder used after twenty-four hours' refrigeration (Table III). Tears were not sterilized.

Tips of sterile Wright's pipettes were macroscopically calibrated to approximately the same interior diameters and marked about 30 mm. from the end. Concentrations of sterile boric acid solutions in distilled water to make desired final concentrations (supersaturated where necessary) were drawn into the pipettes by capillary action up to the mark. This was repeated with tears and again with a twenty-four-hour brain-heart infusion broth culture of the desired organism. Each portion was aspirated to empty the tip for the next portion and aspirated vigorously to mix after the final portion.

TABLE I.—BACTERIAL SENSITIVITY TO BORIC ACID

Boric Acid, %	Plate Counts[a]								
	M. pyogenes var. *albus*			*Streptococcus sp. beta*-Hemolytic			*Diplococcus pneumoniae*		
	Hours			Hours			Hours		
	3	6	24	3	6	24	3	6	24
0.0	1	+	TNTC	TNTC	TNTC	TNTC	++	+++	+++
0.25	6	10	++½	++	+++	TNTC	++	++½	++
0.5	7	7	19	½	++	TNTC	++	+½	½
1.0	3	5	1	+½	+½	++	++	+	0
2.0	2	0	1	++	½	7	++	½	1
3.0	0	0	1	+	22	2	15	3	0
4.0	1	1	0	½	7	1	1	0	0

[a] TNTC = Too numerous to count (over 400); + represents approximately 100 colonies; ½ represents approximately 50 colonies; 0 indicates no growth.

TABLE II.—ANTIBACTERIAL ACTION OF BORIC ACID IN FRESHLY COLLECTED TEARS

Boric Acid, %	Plate Counts[a]					
	M. pyogenes var. *albus*			*Streptococcus sp. beta*-Hemolytic		
	H_2O Control	Tears		H_2O Control	Tears	
		Set 1	Set 2		Set 1	Set 2
0.5	TNTC	+++½	+++	..	..	..
1.0	+++½	+++	++	TNTC	++½	++½
2.0	+++	++½	+½	TNTC	TNTC	TNTC
3.0	..	..	..	TNTC	TNTC	+++

[a] TNTC = Too numerous to count; + represents approximately 100 colonies; ½ represents approximately 50 colonies

TABLE III.—ANTIBACTERIAL ACTION OF BORIC ACID IN REFRIGERATED TEARS

Boric Acid, %	Plate Counts[a]					
	M. pyogenes var. *albus*		*Streptococcus sp. beta*-Hemolytic		*Diplococcus pneumoniae*	
	H_2O Control	Tears	H_2O Control	Tears	H_2O Control	Tears
0.5	TNTC	+++	..	..	..	..
1.0	+++½	++½	TNTC	TNTC	+	½
2.0	+++	++	+++	+++	+	½
3.0	..	..	+++	++½	2	?

[a] TNTC = Too numerous to count; + represents approximately 100 colonies; ½ represents approximately 50 colonies.

The end of the pipette was sealed by flaming and was incubated at 37° for twenty-four hours.

After incubation, the tips of the pipettes were flamed and broken off, the cultures expelled into sterile Petri plates, and brain-heart infusion agar pour plates made. Colonies were counted after forty-eight hours incubation at 37°. Since the tears were not sterilized and contained a normal flora, which consisted mainly of *M. pyrogenes* var. *aureus*, counts were made only of typical colonies of the added bacterial species and normal flora ignored where distinguishable. Results are shown in Tables II and III.

RESULTS

Freshly collected pooled tears seemingly do not interfere with the action of boric acid. Counts shown in Table II tend to be lower in cultures incubated in the presence of tears than in the controls. This was more evident with *M. pyogenes* var. *albus* than with *Streptococcus sp.*

Tears refrigerated for twenty-four hours at 6° showed similar results (Table III) to those obtained with fresh tears.

CONCLUSIONS

Bacteriostatic concentrations of boric acid in broth cultures of representative pyogenic bacterial species often found in the eye varied from 0.5 per cent to 2 per cent. Higher concentrations to a maximum of 4 per cent demonstrated a slow bactericidal action.

Freshly collected as well as stored lacrimal secretions showed no inhibitory action on the antibacterial action of boric acid. The results suggest that tears actually increase the bactericidal action of boric acid to a slight degree. This may be due to naturally occurring lysozyme.

Antifungal Properties of Some Antihistamines*,†

By G. C. WALKER, H. GEORGE DeKAY, and C. L. PORTER

Several antihistamine drugs have been tested for fungistatic activity. By use of the isolated mycelial disk technique, an exposure of two minutes, and *T. mentagrophytes* as the test organism, none of the compounds showed activity. When tested by the agar cup-plate method and the same organism, all of the antihistaminic drugs tested showed inhibitory activity.

IT HAS long been recognized that the dermatophytes produce allergic manifestations within the tissues of the host, some of them severe in nature. It is possible that histamine may be concerned in these tissue reactions, and if such is the case then the antihistamine drugs may prove of value from an antiallergenic standpoint. In addition, any local anesthetic activity they may possess would be useful for the amelioration of the itching associated with athlete's foot.

An examination of the chemical structure of a number of antihistamines has shown the presence of certain radicals such as benzyl and pyridine, which from recent investigations in our laboratories have been found to confer fungicidal and fungistatic properties by virtue of their presence in the molecule. It has recently been shown that three compounds possessing antihistaminic properties also exhibit fungistatic activity (1).

A number of antihistamines were secured from their respective manufacturers and evaluated for fungicidal and fungistatic activity.[1] They are as follows: Antergan hydrochloride, Antistine hydrochloride, Benadryl hydrochloride, Tagathen citrate, Neo-Antergan acid maleate, Neohetramine hydrochloride, Phenergan hydrochloride, Pyribenzamine hydrochloride, and Thephorin hydrogen tartrate.

FUNGICIDAL TESTS

The compounds were evaluated for fungicidal activity using the method of Golden and Oster (2), except for the introduction of polyethylene glycol 400 in the rinsing phase. *Tricophyton mentagrophytes* was grown for fifteen days on Sabouraud's dextrose agar containing 10% normal horse serum. A 1-cm. disk was cut from the agar by means of a

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† Presented before the Subsection on Pharmacy, American Association for the Advancement of Science, Scientific Section, Cleveland, Ohio, December, 1950.

[1] The authors wish to thank the following companies for supplying the antihistamines: Ciba Co.; Hoffmann-La Roche; Lederle Laboratories; Nepera Chemical Co., Inc.; Parke, Davis and Co.; and Poulenc, Ltd.

sterile cork borer and immersed for two minutes in 10 ml. of a 95% alcohol solution of the substance being tested. Following exposure, the disk was rinsed for five minutes with gentle shaking in 10 ml. of a 60% aqueous solution of polyethylene glycol 400, followed by five minutes in 10 ml. of 30% acetone solution, and finally for five minutes in 10 ml. of sterile broth. The disk was then planted, mycelial surface down, on sterile slants of Sabouraud's agar and observed for growth after twenty-one days.

None of the antihistamines tested showed any toxic properties after an exposure of two minutes, but growth of the organism was retarded in some cases for as long as ten days, thus indicating penetration of the agar and fungistatic activity.

FUNGISTATIC TESTS

Preliminary experiments, using the agar cup-plate method of Burlingame and Reddish (3) and *Trichophyton mentagrophytes* as the test organism, showed that all the antihistamines exhibit inhibitory properties to some degree when tested at a concentration of 0.1% in 95% alcohol. It was decided to evaluate them in an ointment base, since it would be in this form of medication that they would most likely appear for the treatment of athlete's foot.

U. S. P. hydrophilic ointment was used with the omission of sodium lauryl sulfate and the substitution of Tween 80 so as to produce a nonionic formulation. The composition was as follows:

Stearyl alcohol	25 Gm.
Glycerin	10 Gm.
Tween 80	3 Gm.
White petrolatum	25 Gm.
Distilled water	37 Gm.

Serum agar plates were prepared containing 10% normal horse serum in Sabouraud's dextrose agar and a further 15 ml. of serum agar containing 2,500,000 conidia from a fifteen-day culture of *Trichophyton mentagrophytes* poured over the surface. A 2-cm. unsealed cup was cut from the center of the agar by means of a sterile cork borer.

The antihistamines were incorporated in the cold base by trituration and in strengths of 2%, 1%, and 0.5%. One and one-half grams of the ointment to be tested was packed into the cup and the plate incubated for five days at 28°. Control tests performed using the ointment base alone showed no fungistatic activity.

Since few definite zones were produced, the results are of necessity descriptive. In practically all the tests, a faint glaze was noted which increased outward from the cup indicating that the organism was partially inhibited. This faint or subdued germination showed little further visible development after an additional observation period of five days. The results are shown in Table I with an antihistamine concentration of 2%. Where a clear-cut zone was evidenced, it was measured in millimeters from the edge of the cup to the circle of mycelial development.

TABLE I.—RESULTS OF FUNGISTATIC TESTS IN A NONIONIC OINTMENT BASE

	Zone of Inhibition
Antergan	7 mm. clear inner zone[a]
Antistine	12 mm. clear inner zone[a]
Benadryl	7 mm. clear inner zone[a]
Tagathen	[b]
Neo-Antergan	[b]
Neohetramine	[b]
Phenergan	[b]
Pyribenzamine	[b]
Theophorin	[b]

[a] Faint visible germination to periphery of plate.
[b] Faint visible germination over entire plate.

Results at 1% and 0.5% showed further visible increases in the extent of germination. Four of the compounds showed greater evidence of inhibition than the others at 0.5%, and the results are descriptively outlined in Table II.

TABLE II.—RESULTS OF FUNGISTATIC TESTS IN A NONIONIC OINTMENT BASE AT A CONCENTRATION OF 0.5%

Antergan	Faint germination outward from the cup for 26 mm., then dense white mycelial growth to periphery
Tagathen	Definite germination for 23 mm., then dense white mycelial growth to periphery
Phenergan	Faint germination outward from the cup for 23 mm., followed by sparse mycelial development to periphery
Thephorin	No distinct zone, but sparse, increasing mycelial development outward from the cup

CONCLUSIONS

1. Using the isolated mycelial disk technique and an exposure of two minutes, the antihistamines tested showed no fungicidal activity toward *T. mentagrophytes*.

2. Results obtained by the agar cup-plate method of fungistatic testing show all the antihistamines tested to possess definite inhibitory activity against *T. mentagrophytes*.

3. Antergan and Phenergan appeared to be the most active of the compounds tested in a nonionic ointment base.

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The Effects of Certain Organic and Inorganic Nitrogen Feedings on Growth and Alkaloid Biosynthesis in *Claviceps* (Ergot) Mycelium*,†

By S. K. SIM‡ and H. W. YOUNGKEN, Jr.§

Ergot alkaloids have been found in young cultures of *Claviceps* mycelia in trace amounts. When cultures were agitated by mechanical shaking, mycelial growth was increased, but alkaloid potency decreased. Supplementary feedings with tryptophane, ornithine, and indole supported alkaloid biosynthesis but markedly inhibited mycelial growth. Arginine and ammonium sulfate had no significant effects on growth or alkaloid synthesis.

THE SCLEROTIUM, or dormant stage, of the fungus *Claviceps purpurea* which is parasitic on the rye plant, constitutes the official drug, Ergot N. F. Several workers have in recent years successfully prepared artificial growth of the mycelial stages of this fungus in laboratory culture media (1–5). McCrea (2) and Jaretzky (3) have demonstrated the presence of the ergot alkaloids in fluidextracts prepared from artificially cultured *Claviceps* mycelia. On the other hand, Michener and Snell (5) have reported that the presence of ergot alkaloids could not be conclusively shown in *Claviceps purpurea* cultures grown under similar conditions as reported above. Furthermore, they reported that when ergotamine had been added to cultures the quantity of this alkaloid was decreased as the incubation period increased.

While the mode of alkaloid formation in *Claviceps* species, as well as in many higher plants, is not clearly understood, in recent decades many studies have been reported on the effects of supplementary controlled nitrogen feedings on alkaloid biosynthesis; for example, the effects of certain amino acids such as arginine and ornithine and inorganic nitrate feedings on alkaloid formation in *Atropa belladonna*, *Hyoscyamus muticus*, and *Datura stramonium* (6–13).

Inasmuch as conflicting reports on alkaloid biosynthesis in *Claviceps* mycelial cultures appear in the literature, it was deemed of interest to re-examine this phenomenon and to ascertain the effects in artificial cultures of controlled nitrogen feedings such as were found to influence growth and alkaloid production in certain higher plants.

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‡ Abstracted from a thesis presented to the Graduate Faculty of the University of Washington by S. K. Sim in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy, May, 1951.

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EXPERIMENTAL

Culture Methods.—Representative dried ergots were selected from two different commercial lots of the drug,[1] and these were dipped into 95% alcohol for about ten seconds in order to prevent the growth of any contaminating organisms. Thereupon, a small piece of the inner tissue of each ergot was carefully dissected from the sclerotium. This tissue was again dipped into 95% alcohol for about five seconds, quickly passed over a Bunsen flame, and immediately planted onto the surface of sterile agar slants. All agar slants were prepared with the formula of McCrea (2) (Table I) and were sterilized by autoclaving at 15 lb. pressure for twenty minutes. These were incubated at room temperature. Mycelial strands appeared on the slant in from ten to fourteen days. A small proportion of the agar slants failed to produce mycelial growth, but growth was successfully induced in most of the cultures. Transplants were made from original agar cultures once every four or five weeks. An authentic strain of *Claviceps purpurea* was also obtained from Dr. E. C. Stakman of the Department of Plant Pathology, University of Minnesota. Growth characteristics of this were compared with those of progenies from the commercial ergot stock and found to be identical. Furthermore, subsequent inoculation of young ovaries of flowering greenhouse rye plants with these cultured mycelial tissues resulted in the formation of an authentic ergot drug.

In order to handle adequately large quantities of the *Claviceps* mycelial tissues for growth measurements and alkaloid studies, it was found necessary to employ liquid culture media. Thus, surface mycelial strands[2] from three- to four-week-old stock culture agar slants were transplanted by means of a standard inoculating needle into standard Kolle culture flasks, 13.5 cm. in diameter, filled with 60 ml. of a sterile liquid medium. The liquid culture medium employed was that successfully used for *Claviceps* growth by Schwarting and Hiner (4) (Table II). McCrea (2) has found that *Claviceps purpurea* grows satisfactorily over a wide temperature range, i. e., from 18° to 28 or 30°. Cultures for this study were maintained at room temperature.

[1] Purchased from Meer's Corporation and the S. B. Penick Company, New York, N. Y.

[2] Grateful acknowledgment is made to Dr. C. M. Wright, Department of Plant Pathology, State College of Washington, for confirming the identification of *Claviceps purpurea* mycelial material employed during the study.

TABLE I.—MCCREA'S FORMULA FOR CLAVICEPS CULTURE (2)

Dihydrogen potassium phosphate	1.25 Gm.
Magnesium sulfate	1.25 Gm.
Peptone (Merck)	1.25 Gm.
Maltose (Difco)	12.50 Gm.
Malt extract (Difco)	12.50 Gm.
Agar (Difco)	60.00 Gm.
Water, sufficient to make	1.00 L.

TABLE II.—SCHWARTING-HINER LIQUID CULTURE FOR CLAVICEPS GROWTH

Magnesium sulfate	1.25 Gm.
Dihydrogen potassium phosphate	2.50 Gm.
Peptone (Merck)	1.25 Gm.
Maltose (Difco)	12.50 Gm.
Yeast extract (Difco)	12.50 Gm.
Water, sufficient to make	1.00 L.

For controlled nitrogen feedings and alkaloid study, two or three experimental groups of cultures were arranged for each feeding growth period. Each experiment consisted of from 15 to 20 flasks per treatment, in addition to a control group consisting of a similar number of flasks. The controls contained 60 ml. of the liquid medium, whereas the treated group consisted of flasks containing this amount with the addition of one of the following nitrogenous compounds: L-arginine, L-ornithine, L-tryptophane, or ammonium sulfate in 0.005 *M* concentration, or indole in concentration of 0.0002 *M*. It had been found in preliminary experiments that higher concentrations of indole greatly inhibited the growth of the fungus, and that with 0.005 *M* or higher concentrations of indole there was no growth at all. All liquid cultures in each experiment were inoculated at the same time with the same stock of *Claviceps* tissue. These subcultures were then allowed to grow side by side at room temperature in the laboratory for seven days and were then harvested simultaneously. In preliminary experiments it was noted also that vegetative mycelial growth was most rapid during a seven- to ten-day growth period.

Harvesting and Drying.—For growth weight data and alkaloid determinations, the pellicles from each flask were collected separately in a tared small evaporating dish which had previously been smeared with a thin film of white petrolatum. The thin film of petrolatum facilitated the removal of the tissue material from the dish after it had been dried. The material was then dried in the oven at 40° for five days, and the dried weight determined for each collection.

Extraction Procedure.—The dried *Claviceps* mycelial tissue was removed from the evaporating dishes, and materials from dishes of the same group in an experiment were then combined. To remove a small unavoidable amount of white petrolatum which inadvertently remained attached to the mycelial tissue, the latter was placed in petroleum ether for about four hours. The petroleum ether was decanted off, and the mycelial material was thoroughly dried in a current of warm air. It was then broken into small pieces. Dried tissues from each group (i. e., 17 to 20 flasks) yielded a dried weight of from 0.5 to 2.0 Gm. This was then extracted with 15–20 ml. of diluted alcohol, U. S. P., containing 1% tartaric acid, and the mixture shaken in a mechanical shaker for twenty-four hours. It was then centrifuged and filtered. The filtrate constituted the fraction which was used for chemical and biological tests. One gram or 0.5 Gm. of previously dried prepared ergot N. F. was extracted in the same manner and at the same time for comparative testing.

Examination for Total Alkaloids.—Extracts which had been prepared in the above manner were first examined qualitatively by a colorimetric procedure. The chemical test of the British Pharmacopœia 1948 (14) which employs the van Urk reagent (*p*-dimethylaminobenzaldehyde) was used. The reagent was freshly prepared before each test by dissolving 0.125 Gm. of *p*-dimethylaminobenzaldehyde in a cooled mixture of 65 ml. of sulfuric acid and 35 ml. of water, and then adding 0.1 ml. of 5% ferric chloride. One milliliter of the mycelial extract was then mixed with 2 ml. of the van Urk reagent. All the extracts from the experimental mycelial tissue materials gave a purplish color which indicated the presence of ergot alkaloids or similar compounds possessing an indole nucleus. After about two to three hours, the color became darker blue. The extract made from prepared ergot N. F. gave a light blue color with the reagent almost immediately. This color also turned very much darker blue on standing. It was noted that the colors developed by the prepared ergot extracts were generally much more intensely blue at first than the response with the mycelial extracts. It was therefore presumed that the quantity of alkaloids present in the experimental mycelial materials was considerably less than that found in prepared ergot N. F. Other compounds having the indole nucleus, in addition to the ergot alkaloids, e. g., indole, tryptophane, and lysergic acid, give this color reaction by the same test. These, however, are not reactive to the biological assay tests in which isolated rabbit uterus muscle strips are employed. Therefore it was feasible to examine for total alkaloids by the use of a modified Broom and Clark (15, 16) uterine strip method.

The biological method of Broom and Clark (15), as modified by Burn (16), makes use of the ability of the ergot alkaloids (ergotoxine and ergotamine) to counteract the contraction caused by a freshly prepared epinephrine solution on isolated rabbit uterus strips. While this method has a disadvantage in that it does not measure the ergonovine content of a preparation, it is sufficiently specific for the ergotoxine type of alkaloids and it requires smaller quantities of material for analysis than does the chemical method. Ergotoxine in quantities of 0.005 to 0.010 mg. (when using a 40–100 ml. muscle chamber) is sufficient for the reaction (16).

Nongravid female rabbits of about 3 Kg. in weight were sacrificed by a blow on the head and bled by cutting the throat. The horns of the uterus were immediately removed and kept in nutrient Tyrode solution (17). A piece of the uterus about 1 to 1.5 cm. long was cut off from one horn. This muscle was then longitudinally divided into two strips and each of these strips was suspended in one of two 40-ml. glass chambers in Tyrode solution. A

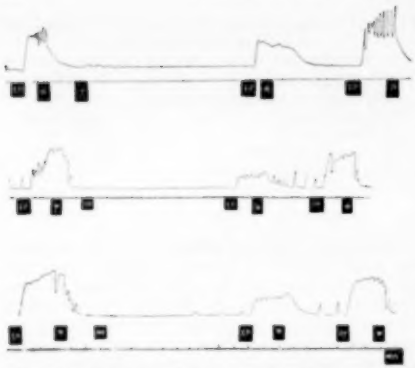

Fig. 1.—Kymograph tracings showing comparative alkaloidal activity in *Claviceps* mycelial tissues grown in culture solutions.

EP = Epinephrine (0.015 γ/ml.); W = Wash; T = Tryptophane-treated culture (1.95 mg./ml.); OR = Ornithine-treated culture (2.06 mg./ml.); N9 = Control culture (2.67 mg./ml.).

All refer to final concentrations of mycelial extract placed into muscle chamber.

constant stream of oxygen (95% with 5% carbon dioxide) was bubbled into the chambers. One end of the uterus muscle strip was securely tied to the hooked end of a suitably bent glass rod clamped to stand vertically in the chamber, and the other end of the strip was tied by thread to a lever above. The lever properly weighted recorded the contraction and relaxation of the muscle strip by tracing on smoked kymograph paper made to rotate slowly around a mechanical drum system (Figs. 1 and 2).

The presence of the alkaloids, ergotoxine and ergotamine, in mycelial cultures was measured by the ability of these extracts to reduce a contraction of the uterus strip produced by 0.5 to 1 ml. of freshly prepared 1:50,000 epinephrine hydrochloride solution. The degree of diminution of the epinephrine contraction was compared to that produced by known quantities of a reference extract of prepared ergot N. F., which had been previously standardized against U. S. P. reference standard ergotoxine ethanesulfonate.

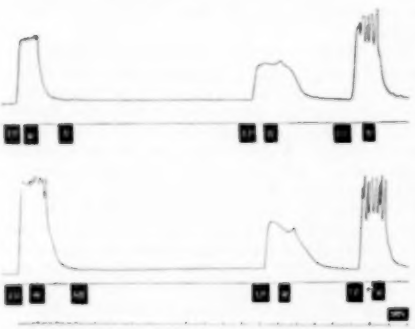

Fig. 2.—Kymograph tracings showing comparative alkaloidal activity in *Claviceps* mycelial tissues grown in culture solutions.

EP = Epinephrine (0.03 γ/ml.); W = Wash; S = Shaken culture (6.68 mg./ml.); N8 = Control culture (0.8 mg./ml.).

All refer to final concentrations of mycelial extract placed into muscle chamber.

The alkaloid content was computed as follows: In each test, after ascertaining the proper amount of epinephrine required to produce a contraction of reproducible magnitude, the muscle strips were washed. In each experiment a suitable quantity, but not more than 2 ml., of mycelial extract was then introduced into one chamber, and an extract of the reference prepared ergot was simultaneously introduced into the second chamber. Ten minutes later, the same amount of epinephrine was again added to each chamber. The presence of ergot alkaloids (as ergotoxine) would cause a diminution of the latter epinephrine contraction. The amount of the extract added in each case was then diluted to determine the minimum quantity necessary to cause a 50–60% diminution of the epinephrine contraction when compared with the initial contraction. This activity was reproduced three or four times before results of the test were concluded. A quantity of mycelial extract was then considered to contain the same amount of alkaloids (as ergotoxine) as a quantity of the prepared ergot extract if it produced the same relative response by causing a 50–60% diminution of the epinephrine contraction. As both of these quantities of the experimental extract and the extract of prepared ergot had been derived from known weights of dried materials (dried mycelia in the one case, and dried prepared ergot in the other), these quantities of the extracts were then converted to their respective dried weight equivalents. That is, if w mg. of dried tissue materials from all the flasks in one group (cultures of same treatment which were inoculated at the same time from the same stock culture as previously described) after extraction yielded an extract of v ml., and v_a ml. of this extract was found to be the minimum quantity necessary to bring about a 50–60% diminution of the epinephrine contraction, then the dried weight of mycelial tissues equivalent to the v_a ml. of extract would be equal to $(w/v) \cdot v_a = M_m$ mg. The dried weight equivalent (M_{pe} mg.) of the extract of prepared ergot was calculated in the same manner. This would mean then that M_m mg. of the experimental mycelia showed the same activity as M_{pe} mg. of prepared ergot. Samples were then considered to contain the same amount of alkaloids.

The total alkaloid content of the prepared ergot used (as mg. ergotoxine per 100 Gm. prepared ergot) was determined by the British Pharmacopœia 1948 (14) colorimetric method as follows: A ten-gram sample of the prepared ergot was defatted with petroleum ether and then extracted with anhydrous ether in a Soxhlet extractor. The ether extract was then shaken with portions of 1% tartaric acid solution. A suitable aliquot of the acid solution was mixed with two volumes of the *p*-dimethylaminobenzaldehyde reagent. The intensity of the color produced was measured at 590 mμ with a Beckman spectrophotometer. The quantity of total alkaloids in the acid solution was determined by comparing the color intensity with that produced by the reaction of the same reagent with standard solutions of U. S. P. reference standard ergotoxine ethanesulfonate in 1% tartaric acid. The results of

TABLE III.—TOTAL ALKALOID ANALYSIS FOR PREPARED ERGOT[a]

Sample of Prepared Ergot	Per Cent Total Alkaloids (as Ergotoxine)
1	0.0875
2	0.0700
3	0.0800
4	0.0600
5	0.0600
6	0.0530
7	0.0625
8	0.0665

Average content of total alkaloids = 0.0674% = 67.4 mg./100 Gm.

[a] Determined by the method of the British Pharmacopœia 1948.

eight assays gave an average alkaloid content of 67.4 mg. (as ergotoxine) per 100 Gm. prepared ergot (Table III).

Statistical Analyses for Dried Weight Yields.—Comparison of dried weights of mycelia were made only between the control group and the treated group or groups in the same experiment. For such comparisons the mean dried weight of mycelial tissues per flask in each group was used. These dried weights were tested for statistical significance by computing the "Student t" (18) and then obtaining from statistical tables (19) with reference to each experiment's particular number of degrees of freedom, the probability point. The formula used for computing the "Student t" follows:

$$t = \frac{M_1 - M_2}{s\sqrt{\frac{1}{N_1} + \frac{1}{N_2}}}$$

where M_1 and M_2 are, respectively, the mean dried weights per flask of the control group and the treated group. N_1 and N_2 are, respectively, the numbers of observations of the two groups, and s represents the standard deviation or the square root of the variance, and is calculated as follows:

$$s = \sqrt{\frac{Sd_1^2 + Sd_2^2}{N_1 + N_2 - 2}}$$

In this formula N_1 and N_2 are as explained above, Sd_1^2 is the sum of squares of deviations of the first group's observations about their mean, and Sd_2^2 is the sum of squares of deviations of the second group's observations about their mean. It will be seen that $(N_1 + N_2 - 2)$ denotes the number of degrees of freedom of that particular experiment.

RESULTS

Growth Findings.—All subcultures maintained on the agar medium, used for stock cultures, grew well. From the point of inoculation, the white, woolly mycelial growth spread to cover the entire surface of the agar slant in about four weeks. But it never penetrated to more than about 0.5 mm. in depth. Aerial mycelia grew to a height of about 1 mm. Conidiospores were formed in four to six days following inoculation. After about ten weeks of growth, the mycelial mat became progressively gray in color, and in about twenty weeks, the culture became noticeably dry. However, sixteen-week-old cultures were sufficiently viable when transplanted to fresh culture medium.

In the experimental series grown on liquid medium, small, dull, opaque, gelatinous pellicles, about 0.5 cm. in surface-area diameter, began to appear on the third or fourth day after inoculation. These floated on the surface of the liquid medium. They soon grew thicker and larger in size, and by the end of seven to ten days, the major surface area of the culture broth was covered with either one continuous pellicle sheet or several pellicles of smaller size. Seven-day-old pellicle when examined under the microscope showed numerous branching long-cell hyphae and many single-cell conidiospores. Following this period, conidiospores became more abundant and mycelial growth failed to increase in proportion. Many yellowish-brown droplets of ergot oil were observed to float on the surface of the pellicle of some three-week-old subcultures. In other cultures, these oil drops were not formed. The *Claviceps* pellicles became gray in color after more than two weeks of growth.

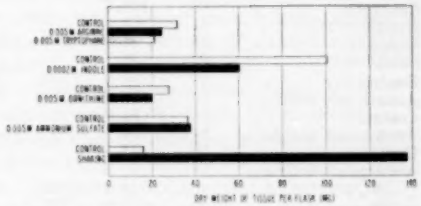

Fig. 3.—Bar graphs showing comparative dried weight yields of *Claviceps* mycelia growth from cultures treated with arginine, tryptophane, indole, ornithine, ammonium sulfate, and mechanical shaking.

Comparison of Dried Weights of Mycelia from Feeding Experiments.—The results of the effects produced on mycelial growth by the feeding experiments, during which the nitrogenous compounds indole (0.0002 *M*), L-arginine (0.005 *M*), L-tryptophane (0.005 *M*), L-ornithine (0.005 *M*), and ammonium sulfate (0.005 *M*) were added to the cultures, are given in Table IV and Fig. 3. Those results marked "a" were statistically analyzed in the manner described. These experiments have been repeated two or three times in each case. The average dried weight per flask was determined by dividing the total dried weight of each group by the number of cultures contained in that group. The results of these experiments are marked "b" and "c" in Table IV. It will be noted that the results of the repeated experiments marked "b" and "c" in Table IV quite parallel those that are marked "a" in the same table in every case except that of the arginine feedings.

It was noted that by comparing statistical results of the dried weights produced by each experimental lot as described above, significant differences in growth could be observed between cultures containing indole, L-tryptophane, L-ornithine, and their respective controls. No significant differences were noted in the case of arginine and ammonium sulfate treatments.

TABLE IV.—DRIED WEIGHTS AND ALKALOID ACTIVITY DATA OF CLAVICEPS MYCELIA GROWN IN CULTURE SOLUTIONS WITH NITROGEN FEEDINGS AND WITH SHAKING

Dried Weights						Alkaloid Activity		
	No. of Flasks	Mean Dried Wt. of Mycelia per Flask, Mg.	Relative Mean Dried Wt. per Flask	"t"	Probability Point		Alkaloid Content Mg. Ergotoxine per 100 Gm. Mycelia	Mean Alkaloid Content Mg. Ergotoxine per 100 Gm. Mycelia
Control a	17	100.6	(100)	3.669	0.001	Control a	0.21	
Indole a	15	60.4	60.05			Control b	0.30	0.225
Control b	17	101.2				Indole a	0.25	
Indole b	21	63.5				Indole b	0.37	0.310
Control a	18	32.3	(100)			Control a	0.44	
Arginine a	18	25.3	78.32			Control b	0.34	0.39
Control b	17	101.2				Arginine a	0.56	
Arginine b	17	100.3				Arginine b	0.60	0.58
Control a	18	32.3	(100)	7.698	<0.001	Control a	0.44	
Tryptophane a	18	21.7	67.18			Control b	0.41	
Control b	19	39.1				Control c	2.26	1.06
Tryptophane b	20	22.8				Tryptophane a	0.65	
Control c	15	24.9				Tryptophane b	0.73	
Tryptophane c	17	19.9				Tryptophane c	2.50	1.30
Control a	19	28.1	(100)	2.813	<0.01	Control a	0.48	
Ornithine a	20	20.1	71.53			Control b	0.25	
Control b	17	100.6				Control c	0.57	0.43
Ornithine b	17	72.9				Ornithine a	0.62	
Control c	15	24.9				Ornithine b	0.37	
Ornithine c	20	22.7				Ornithine c	0.75	0.58
Control a	18	36.9	(100)	0.1346	0.5	Control a	0.43	
Ammonium sulfate a	18	37.5	101.6			Control b	0.57	
Control b	15	24.9				Control c	0.48	0.49
Ammonium sulfate b	14	24.4				$(NH_4)_2SO_4$ a	0.38	
Control c	19	28.1				$(NH_4)_2SO_4$ b	0.62	
Ammonium sulfate c	19	26.6				$(NH_4)_2SO_4$ c	0.56	0.52
Control a	10	16.1	(100)	6.573	<0.001	Control a	1.59	
Shaking a	10	137.2	852.1			Control b	2.41	
Control b	10	28.0				Control c	1.52	1.84
Shaking b	10	204.3				Shaking a	0.19	
Control c	10	19.9				Shaking b	0.44	
Shaking c	10	119.3				Shaking c	0.64	0.42

Indole feeding produced a decrease in the average dried weight of approximately 40% when compared with its control. Although arginine feeding resulted in a 22% decrease in dried weight in the experiment marked "Arginine a" in Table IV, there appeared to be no such difference in the results marked "Arginine b."

The response to tryptophane feeding in the experiment marked "Tryptophane a" showed that there was about 33% less mycelial dried weight yield than in the case of the control. Ornithine, when added to the culture solutions, produced an average decrease of dried weight of approximately 28.5% as compared with the controls ("Ornithine a" in Table IV). The response from feedings in which ammonium sulfate (0.005 *M*) was employed revealed that this form of inorganic nitrogen produced no significant effect on mycelial growth.

Aeration-Agitation of Claviceps Cultures by Shaking.—Aeration-agitation has been known to be influential in the metabolic activities of mold cultures from all aspects (20). Hence it was of interest to determine whether or not shaking the *Claviceps* subcultures would influence the growth or total alkaloid production or both.

Each experiment consisted of two groups. Each group consisted of ten 500-ml. Erlenmeyer flasks containing 60 ml. of liquid culture medium. These were autoclaved and inoculated with surface mycelium from a stock culture agar slant in the manner previously described. One of the two groups was continuously shaken by a mechanical shaker throughout the growth period, which lasted seven days. The other group (control) was not shaken but was allowed to grow for the same period. Harvesting, extraction, and chemical and biological tests were carried out in the manner already described. The results (Table IV and Fig. 3) showed that the shaking increased the average dried weight of mycelial tissue per flask by some eightfold.

Total Alkaloid Content of Claviceps Mycelia.—The results of the biological tests of the experimental *Claviceps* mycelia for total alkaloid contents are listed in Table IV. It will be seen that the alkaloid contents of the *Claviceps* mycelia from the control, as well as from the treated cultures, were very meager compared with the total alkaloid content of prepared ergot (Table III) which is derived from *Claviceps* sclerotia. Because of the small alkaloid content of the mycelia (which was from $^1/_{40}$ to $^1/_{200}$ of that of prepared ergot), it has not been possible to assay the mycelial tissue material from each culture flask. As has been pointed out, the mycelial dried weight data show that the addition of indole,

tryptophane, and ornithine to the culture solutions resulted in significant decreases in dried weight. From the analyses for total alkaloids, it was observed that these feedings did not decrease the alkaloid contents of the mycelia despite the significant decrease in mycelial growth. In fact, the results of the assays of the individual groups of treated cultures gave evidence that there was an increase in alkaloid content of the myclelia as compared with the mycelia from the respective controls.

When cultures of mycelial growth were shaken, there was an increase in dried weight. On the other hand, shaking in this case appeared to have effected a considerable decrease in total alkaloid content. When the alkaloid data of the six pooled groups (Shaking a, b, and c and their controls in Table IV) were analyzed statistically, the "t" value was found to be 4.522 with four degrees of freedom and a probability point of 0.01, which indicated a significant decrease in total alkaloid production (as ergotoxine) as a result of the shaking.

DISCUSSION

The results of these experiments show that *Claviceps* cultures grown on the same liquid medium but inoculated from different stock culture agar slants and at different times, varied in growth. This made it necessary to maintain one control group of cultures for each experiment.

The results of alkaloid analyses have confirmed the findings of McCrea (2) and Jaretzky (3), who reported that alkaloids were present in mycelial tissues of *Claviceps purpurea*.

The combined tissue material from each culture group showed an increase of alkaloids in mycelia treated with certain organic nitrogen compounds. However, the alkaloid content of mycelial tissue of each single culture flask of each group was insufficient for adequate quantitative comparisons. Therefore, the significance of these differences in alkaloid production between the treated mycelia and mycelia from untreated cultures cannot be conclusively analyzed by statistical methods under the conditions herein described.

Foster (20) has indicated the very high sensitivity of fungi in general to slight variations of nitrogen source and trace element nutrition. The decreases in dried weight of the mycelia in experimental *Claviceps* cultures resulting from the addition of small concentrations of indole, tryptophane, and ornithine indicate that the growth of this fungus might be influenced by its sensitivity to the concentrations used, but alkaloid production is at the same time not curtailed. The indications, as shown by James (6–8), that the greatest production of alkaloids in higher plants (*Atropa* and *Hyoscyamus*) occurs in the actively growing meristematic tissues, do not seem to apply to the *Claviceps* fungus. But the indications that increased alkaloid production accompanies protein breakdown in the case of *Atropa belladonna* leaves (7, 8) may perhaps also hold true in the *Claviceps* mycelia in culture.

Total alkaloid production (as ergotoxine) in *Claviceps purpurea* mycelial tissues under the conditions described is not sufficient for practical purposes. However, the possibility that increased nitrogen feedings in combination with a continuous shaking procedure might overcome some of the difficulties cannot be overlooked. This phase of the problem is being continued.

SUMMARY

A study has been made of the growth and alkaloid production (as ergotoxine) in the mycelial tissues of the fungus *Claviceps purpurea* when grown in modified culture media.

1. Ergot alkaloids were found to be present in small amounts in all of the mycelial cultures that were examined.

2. The sclerotium stage (as prepared ergot) yielded from 40 to 250 times more total alkaloids (measured as ergotoxine activity) than *Claviceps* mycelial tissues.

3. The addition of small quantities of indole (0.0002 *M*), ornithine (0.005 *M*), and tryptophane (0.005 *M*) to a standard culture medium resulted in a decrease of mycelial growth. On the other hand, each of these feedings showed evidence for some increase in alkaloid content of mycelia.

4. The addition of arginine (0.005 *M*), and of ammonium sulfate (0.005 *M*) produced no significant responses in either growth or alkaloid content in the mycelial tissues of this fungus.

5. Mechanical shaking of the cultures increased the dried weight of the *Claviceps* mycelia some eightfold. This increase in growth was accompanied by a marked decrease in alkaloid content.

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Reduction of Alloxan in Aqueous Solution. II. Effect of Certain Organic Reductants at *p*H 1.6*

By R. STUART TIPSON and LEONARD H. CRETCHER†

By use of ultraviolet absorption spectra, it is found that *l*-cysteine, glutathione, and L-ascorbic acid have no reducing effect on alloxan in aqueous solution at *p*H 1.6.

ALMOST immediately after the discovery that administration of sufficient aqueous alloxan solution induces an experimental diabetes in animals (1), it was surmised that endogenous alloxan might be connected with the cause of human diabetes mellitus (2). In order to test this hypothesis, it became necessary to examine the tissues and fluids of animals (diabetic and nondiabetic) for its possible presence. In developing a suitable procedure, we must first be cognizant of the transformations which alloxan is capable of undergoing in aqueous solution under various conditions; second, we must devise ways of extracting tissues and treating fluids such that their endogenous alloxan content (if any) is not subjected to conditions which would lead to its transformation or decomposition.

A review of the literature made it apparent that, if the biological material is to be exposed to the air, it should be brought to a *p*H below 3.5 as rapidly as possible. Should it contain endogenous alloxan as such, there will then be no transformation to alloxanic acid; should endogenous alloxan be present in its reduced form (dialuric acid), oxidation to alloxan may occur, but there will be no transformation of the latter to alloxanic acid. There then remains the problem of identifying (and, if possible, determining quantitatively) the alloxan or dialuric acid, probably present in the resulting solution in very low concentration.

One promising method for accomplishing this appeared to lie in the examination of the ultraviolet absorption spectra of aqueous solutions, first under reducing conditions and then under oxidizing conditions. If dialuric acid is already present, or is formed by reduction of alloxan, a peak at 270 mμ in the ultraviolet absorption spectrum (3) should be observable. On the other hand, under oxidizing conditions, the alloxan already present, or formed by oxidation of dialuric acid, will exhibit no such peak, although it will display a characteristic ultraviolet absorption spectrum (4).

According to the literature, alloxan in aqueous solution is reduced by excess cysteine (5–10), glutathione (9–11), or L-ascorbic acid (7, 12, 13). Although in some cases (9, 10) reduction was accomplished at *p*H 7.4, in others the *p*H was not always precisely indicated; but it was apparent that some of these reductions were conducted in acid solution. [We have repeated Abderhalden's experiment (12) and have found that, immediately after admixture, the reaction solution has *p*H 2.55.] This inference was strengthened by the knowledge that alloxan is highly unstable in neutral or alkaline solutions (14). Consequently, in all the experiments to be reported, 0.03 *N* aqueous hydrochloric acid (*p*H 1.60) was used as the solvent; in the wave length range employed (220 to 320 mμ), its ultraviolet absorption spectrum is indistinguishable from that of distilled water.

The ultraviolet absorption spectra of various concentrations of alloxan (Fig. 1) and of L-ascorbic acid (Fig. 2) in this solvent were first determined. The results agreed qualitatively with those previously recorded for alloxan (4, 15) and for L-ascorbic acid (16) in somewhat similar solvents. Moreover, within the limits of experimental error, Beer's law was obeyed. Next, the effect of adding, to a known volume of fresh 0.0002 *M* alloxan solution, an equal volume of fresh 0.0002 *M* L-ascorbic acid solution in presence of air was studied. The ultraviolet absorption spectrum of the resulting solution (Fig. 3) was indistinguishable from that calculated by summation of the curves for 0.0001 *M* alloxan and 0.0001 *M* L-ascorbic acid; it did not agree with that calculated for 0.0001 *M* dialuric acid plus 0.0001 *M* dehydro-L-ascorbic acid. The effect was similar when equal volumes of fresh 0.0001 *M* (or of 0.00005 *M*) solutions of alloxan and L-ascorbic acid, respectively, were united (Fig. 4). In no case was the formation of material exhibiting a peak at 270 mμ observed.

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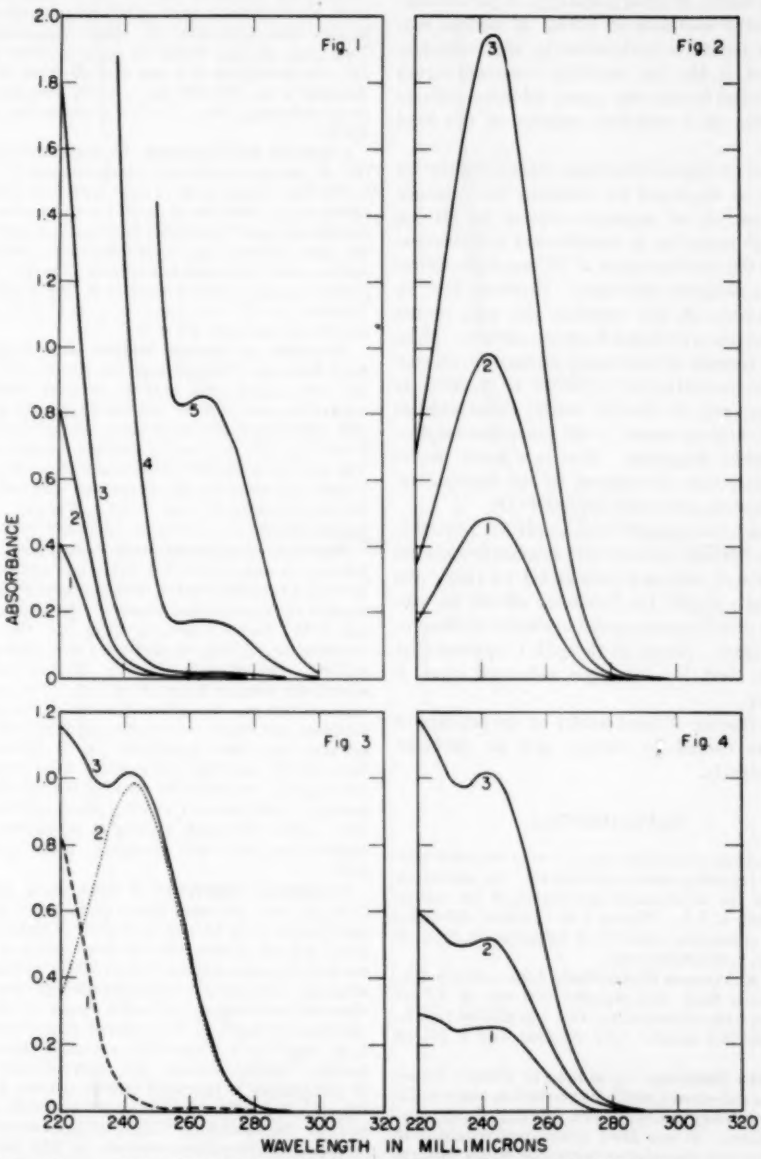

Fig. 1.—Alloxan. (*1*) 0.00005 *M*; (*2*) 0.0001 *M*; (*3*) 0.0002 *M*; (*4*) 0.002 *M*; (*5*) 0.01 *M*.
Fig. 2.—L-Ascorbic acid. (*1*) 0.00005 *M*; (*2*) 0.0001 *M*; (*3*) 0.0002 *M*.
Fig. 3.—(*1*) 0.0001 *M* alloxan; (*2*) 0.0001 *M* L-ascorbic acid; (*2*) 0.0001 *M* alloxan in 0.0001 *M* L-ascorbic acid.
Fig. 4.—(*1*) 0.000025 *M* alloxan in 0.000025 *M* L-ascorbic acid; (*2*) 0.00005 *M* alloxan in 0.00005 *M* L-ascorbic acid; (*3*) 0.0001 *M* alloxan in 0.0001 *M* L-ascorbic acid.

Similarly, when equal volumes of fresh solutions of 0.0001 *M* alloxan and 0.01 *M* glutathione, or of fresh solutions of 0.0002 *M* alloxan and 0.01 *M* *l*-cysteine hydrochloride, were mixed in presence of air, the resulting observed curves (Figs. 6 and 9) were also purely additive, with no indication of a chemical reaction of the kind sought.

Hence, it appears that these organic reductants cannot be employed for revealing the presence of alloxan in an aqueous solution at *p*H 1.6 (through reduction to dialuric acid and observation of the resultant peak at 270 mμ in the ultraviolet absorption spectrum). However, even in the presence of, say, cysteine, this peak results on reduction with excess hydrogen sulfide. Thus, in the absence of interfering substances, alloxan in a concentration of 0.000025 to 0.00005 *M* (4–8 μg./ml.) is thereby readily detectable as dialuric acid by means of the particular spectrophotometer employed. This is a much poorer sensitivity than is achieved by the fluorometric procedure we previously reported (13).

It has been observed that, at *p*H 3.2, *l*-cysteine does not protect dialuric acid solution from aerial oxidation as well as it does at *p*H 7.4 (15). We find that, at *p*H 1.6, *l*-cysteine affords no protective action against aerial oxidation of alloxantin solution. Hence, at this *p*H, it appears that dialuric acid is a stronger reductant than *l*-cysteine.

The bearing of these results on the problem of diabetes caused by alloxan will be discussed subsequently.

EXPERIMENTAL

Ultraviolet absorption spectra were obtained with a Cary recording spectrophotometer; the maximum error in the wave-length calibration of the instrument was ± 5 Å. Figures 1 to 12 record the ultraviolet absorption spectra of solutions in 0.03 *N* aqueous hydrochloric acid.

0.03 *N* Aqueous Hydrochloric Acid.—Into a 1-L. volumetric flask was pipetted 300 ml. of 0.1 *N* aqueous hydrochloric acid; this was diluted to 1 L. with distilled water. The solution had a *p*H of 1.60.

Alloxan Solutions.—A sample of alloxan monohydrate was chosen which was colorless, and readily and completely soluble in five volumes of cold, distilled water. It was dried to constant weight over soda lime and phosphorus pentoxide in the vacuum desiccator at room temperature, and then assayed 99–100% alloxan monohydrate by Tipson and Cretcher's method (13). A stock solution (0.2 *M*) was prepared fresh each day from this alloxan monohydrate (0.3202 Gm.) made up to 10.0 ml. with 0.03 *N* hydrochloric acid; solutions of desired concentration were obtained by suitable dilution of aliquots with the same solvent. The ultraviolet absorption spectra of solutions of various concentrations are shown in Fig. 1. An alloxan solution (0.01 *M*) exhibited a peak at 265 mμ (ε = 85) which became less noticeable at lower concentrations. This peak was not noted by early workers (3, 17, 18); an indication of it was first observed (4) as a shoulder at ca. 255–275 mμ (ε = ca. 70), and later as an inflection (9) at 270 mμ (ε about 90), below *p*H 5.0.

L-Ascorbic Acid Solutions.—A fresh stock solution (0.1 *M*) was prepared from L-ascorbic acid (U. S. P.; 0.1761 Gm.) made up to 10.0 ml. with 0.03 *N* hydrochloric acid; solutions of desired concentration were rapidly prepared by suitable dilution of aliquots with the same solvent, and their ultraviolet absorption spectra were immediately recorded (Fig. 2). These showed a peak at 242.5 mμ (ε = 9,750) at *p*H 1.60. [Herbert, *et al.* (16), found ε = ca. 7,500 at 245 mμ (in aqueous acid, *p*H < 3).]

Admixture of Alloxan Solution with L-Ascorbic Acid Solution.—Alloxan solution (0.0002 *M*; 10.0 ml.) was mixed with 10.0 ml. of fresh 0.0002 *M* L-ascorbic acid solution, and the ultraviolet absorption spectrum immediately observed (curve *3*, Figs. 3 and 4). No attempt to exclude air was made. The spectra of 50:50 mixtures of 0.0001 *M* (and of 0.00005 *M*) solutions of alloxan and L-ascorbic acid are also recorded (curves *2* and *1*, Fig. 4). There was no indication of formation of dialuric acid.

Repetition of Abderhalden's Experiment (12).—Alloxan monohydrate (2.0 Gm.) was dissolved in 60.0 ml. of distilled water, with gentle stirring. As soon as the crystals had dissolved, the solution had *p*H 3.70. Next, L-ascorbic acid (2.0 Gm.) was dissolved in 40.0 ml. of distilled water, with gentle stirring. Immediately after the crystals had dissolved, the solution had a *p*H of 2.45. The ascorbic acid solution was immediately added to the alloxan solution; the mixed solution had a *p*H of 2.55. The solution was now transferred to an Erlenmeyer flask which was then stoppered. After elapse of ten minutes, deposition of colorless crystals became evident; their amount steadily increased for some time. After standing overnight (stoppered), the supernatant liquor was decanted; it had a *p*H of 2.25.

Glutathione Solutions.—A fresh stock solution (0.2 *M*) was prepared from glutathione (0.6146 Gm.) made up to 10.0 ml. with 0.03 *N* hydrochloric acid; 5.0 ml. of this solution was diluted to 100.0 ml. with the same solvent, to give 0.01 *M* glutathione solution. The ultraviolet absorption spectrum was observed immediately and after elapse of five and twenty-four hours in a stoppered flask (curves *1*, *2*, *3*; Fig. 5); it is seen that a gradual change occurred. Similar behavior was observed with 0.005 *M* glutathione in the same solvent (curves *4*, *5*, *6*; Fig. 5); it is probably occasioned by aerial oxidation to the disulfide. The peaks recorded (19) for aqueous glutathione solution (at 252, 280, and 325 mμ) were not noticeable at the dilutions here employed.

Admixture of Alloxan Solution with Glutathione Solution.—Alloxan solution (0.0001 *M*; 10.0 ml.) was mixed with 10.0 ml. of fresh 0.01 *M* glutathione solution, and the ultraviolet absorption spectrum observed immediately and after elapse of five and twenty-four hours in a stoppered flask (curves

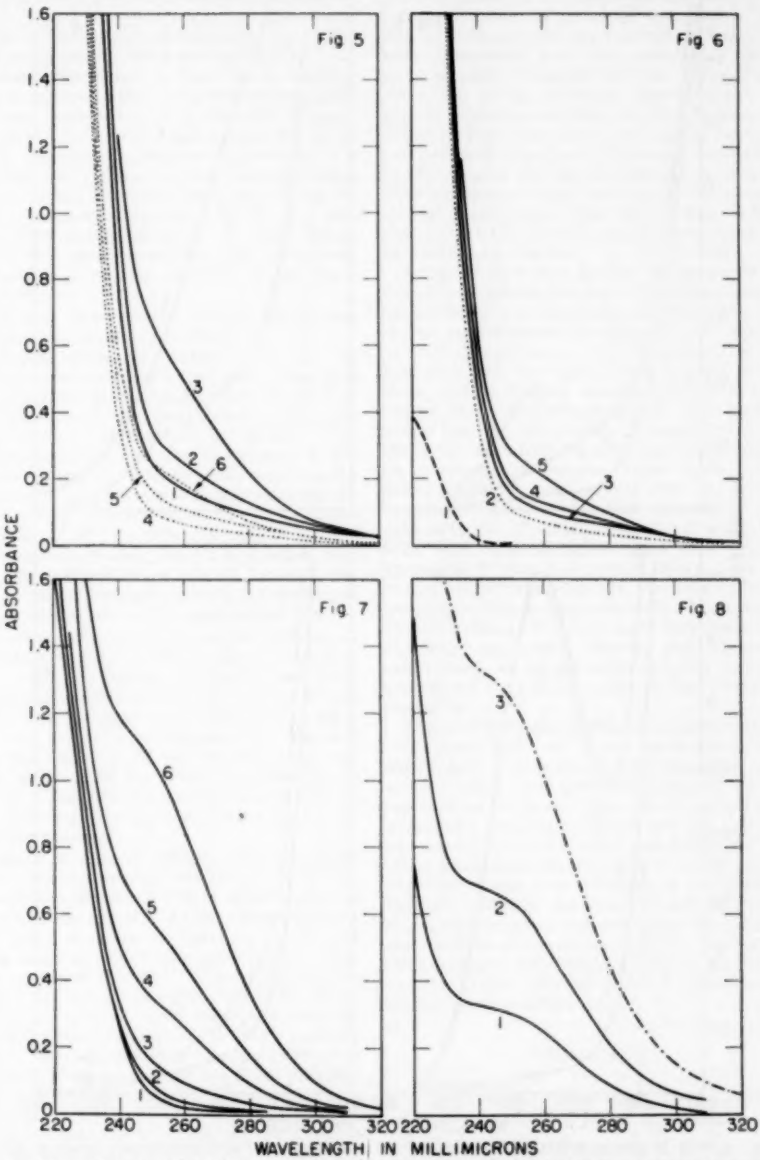

Fig. 5.—Glutathione. 0.01 *M* (*1*) immediate; (*2*) after five hours; (*3*) after twenty-four hours at 25° 0.005 *M* (*4*) immediate; (*5*) after five hours; (*6*) after twenty-four hours at 25°.

Fig. 6.—(*1*) 0.00005 *M* alloxan; (*2*) 0.005 *M* glutathione. 0.00005 *M* alloxan in 0.005 *M* glutathione; (*3*) immediate; (*4*) after five hours; (*5*) after twenty-four hours at 25°.

Fig. 7.—0.01 *M* *l*-cysteine. (*1*) immediate; (*2*) after two hours; (*3*) five and one-half hours; (*4*) twenty-four hours; (*5*) forty-eight hours; (*6*) one hundred and forty-four hours at 25°.

Fig. 8.—*l*-Cystine. (*1*) 0.001 *M*; (*2*) 0.002 *M*; (*3*) saturated at 25°.

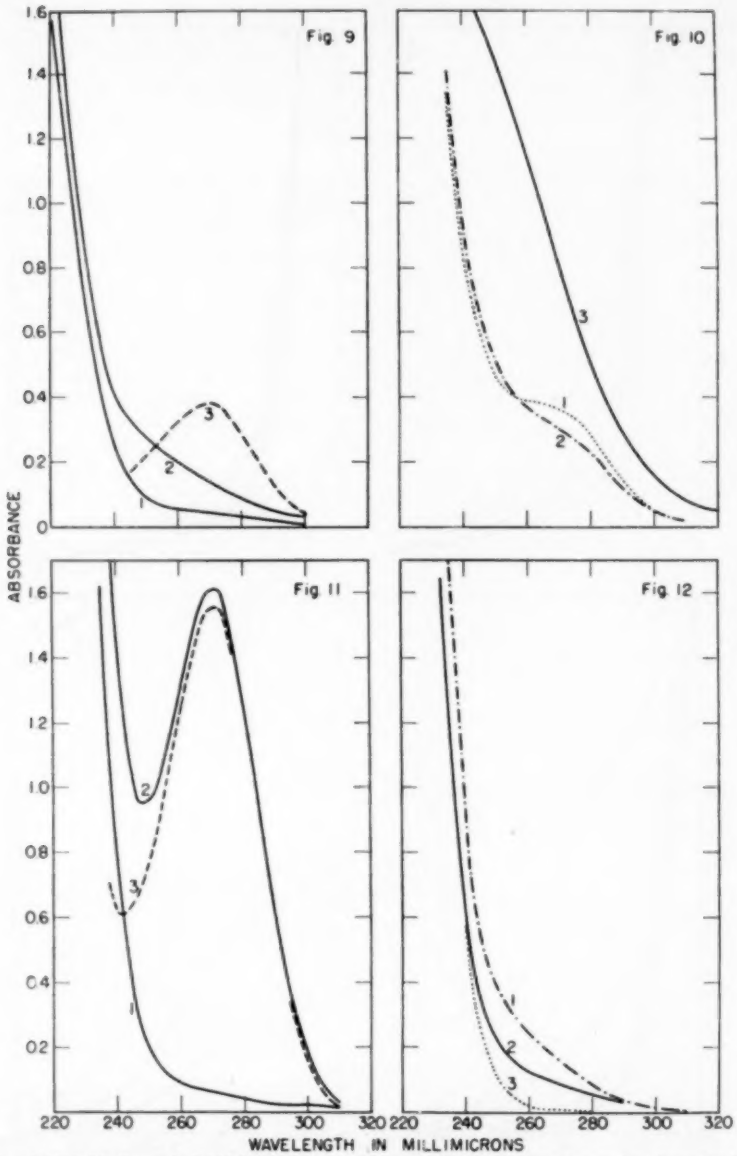

Fig. 9.—0.0001 *M* alloxan in 0.005 *M* *l*-cysteine. (*1*) immediate; (*2*) after twenty-four hours at 25°. (*3*) Calculated, had complete reduction of alloxan occurred.

Fig. 10—0.0002 *M* alloxantin in 0.01 *M* *l*-cysteine, after (*1*) ninety minutes; (*2*) five hours; (*3*) one hundred and forty-four hours at 25°.

Fig. 11—0.03 *N* hydrochloric acid solutions saturated with hydrogen sulfide (*1*) 0.01 *M* *l*-cysteine; (*2*) 0.0002 *M* alloxantin (originally) in 0.01 *M* *l*-cysteine, minus absorbance of colloidal sulfur; (*3*) curve *2*, minus curve *1*.

Fig. 12.—Effect of excess hydrogen sulfide on ultraviolet absorption spectrum of *l*-cystine (0.001 *M* in 0.03 *N* hydrochloric acid. Saturated with hydrogen sulfide). (*1*) Immediate; (*2*) after twenty-eight and one-half hours (minus absorbance of colloidal sulfur); (*3*) after one hundred and seventy-one hours at 25°.

3, 4, 5; Fig. 6); it is seen that a gradual change, presumably attributable to oxidation of glutathione, occurred. At no time was there any indication of formation of dialuric acid, or of combination (9, 10) to give a substance exhibiting a peak at 305 mμ.

***l*-Cysteine Solutions.**—A fresh stock solution (0.2 *M*) was prepared from *l*-cysteine hydrochloride (0.3152 Gm.) made up to 10.0 ml. with 0.03 *N* hydrochloric acid; 5.0 ml. of this solution was diluted to 100.0 ml. with the same solvent, to give 0.01 *M* *l*-cysteine solution. The ultraviolet absorption spectrum was recorded immediately (curve *1*, Fig. 7) and after various time intervals (Fig. 7); it is seen that a gradual change occurred. In order to find out if this was due to aerial oxidation to *l*-cystine, the ultraviolet absorption spectrum of the latter was determined.

The (initial) spectrum of *l*-cysteine agreed with that recorded in the literature (20, 21).

***l*-Cystine Solutions.**—A solution (0.002 *M*) was prepared by placing 0.04806 Gm. of *l*-cystine in a 100-ml. volumetric flask, making up to 100 ml. with 0.03 *N* hydrochloric acid, and stirring magnetically until the solid had dissolved (ninety minutes). The ultraviolet absorption spectrum of the solution was then observed (curve *2*, Fig. 8). The spectrum of a 0.001 *M* *l*-cystine solution was similarly determined (curve *1*, Fig. 8). These spectra show an indication of the peak at 251 mμ previously recorded (21); they were unchanged after one, ten, and sixteen days at 25°. [It has been stated (22) that cystine, dissolved in 0.025 *N* hydrochloric acid, is transformed to cysteine after seven days at room temperature.]

It was then possible to ascertain the solubility of *l*-cystine in 0.03 *N* hydrochloric acid at room temperature (25°), as follows. *l*-Cystine (0.1 Gm.) was suspended in 100 ml. of 0.03 *N* hydrochloric acid and stirred magnetically, from time to time, during three days at room temperature. The suspension was then filtered and the ultraviolet absorption spectrum of the filtrate observed (curve *3*, Fig. 8). The solution proved to be 0.004 *M*; hence, the solubility is ca. eight times that in distilled water (23) at 19°.

Admixture of Alloxan Solution with *l*-Cysteine Solution.—Alloxan solution (0.0002 *M*; 10.0 ml.) was mixed with 10.0 ml. of 0.01 *M* cysteine solution (25 times the theoretical), and the ultraviolet spectrum observed immediately (curve *1*, Fig. 9) and after the elapse of twenty-four hours (curve *2*, Fig. 9) in a stoppered flask at 25°. The curve calculated for 0.0001 *M* dialuric acid plus 0.0001 *M* cystine plus 0.0048 *M* cysteine, which would have resulted had complete reaction occurred, is shown as curve *3* (Fig. 9). Similar effects were observed when 10.0 ml. of 0.0001 *M* alloxan solution was mixed with 10.0 ml. of 0.01 *M* *l*-cysteine solution (50 times the theoretical); again, there was no indication of a reaction (except for noticeable oxidation of the cysteine after twenty-four hours at 25°).

Aerial Oxidation of Alloxantin Dissolved in *l*-Cysteine Solution.—Finely powdered alloxantin dihydrate (0.06444 Gm.) was made to 1 L. with fresh 0.01 *M* *l*-cysteine (in 0.03 *N* hydrochloric acid), and the suspension stirred magnetically in the presence of air until the solid had dissolved. The ultraviolet absorption spectrum of this supposed 0.0002 *M* alloxantin solution was then immediately recorded (ninety minutes after initial admixture), and after the elapse of five hours and one hundred and forty-four hours (after initial admixture). From Fig. 10, it is seen that the vestigial peak at 270 mμ soon disappeared, and then oxidation of the cysteine to cystine became evident. Subtraction of the values on the curves for correspondingly aged 0.01 *M* *l*-cysteine solutions (see Fig. 7) from those shown in Fig. 10 reinforced the former conclusion, and indicated oxidation of the alloxantin to alloxan. In other words, the cysteine afforded no protective influence against aerial oxidation of the alloxantin during dissolution of the latter. This indicates that, at *p*H 1.6, dialuric acid is a more potent reductant than is cysteine.

Action of Hydrogen Sulfide on Alloxantin Dissolved in *l*-Cysteine Solution.—Simultaneously with the preceding experiment, the ultraviolet absorption spectra were measured for the 0.0002 *M* alloxantin (in 0.01 *M* cysteine in 0.03 *N* hydrochloric acid) saturated with hydrogen sulfide at room temperature, and for 0.01 *M* cysteine (in 0.03 *N* hydrochloric acid) similarly saturated with hydrogen sulfide (see Fig. 11). Subtraction of the values on the latter curve from those on the former, followed by subtraction of the absorbance of the colloidal sulfur produced, gave curve *3* (Fig. 11) which is practically identical with that obtained on treatment of 0.0002 *M* alloxantin (in 0.03 *N* hydrochloric acid) with excess hydrogen sulfide (24). There is thus no doubt that, had cysteine been capable of reducing alloxantin at this *p*H, the peak at 270 mμ would have been readily observable. After keeping these solutions at 25° for six days, their spectra were practically unchanged, showing that (*a*) dialuric acid is stable at this *p*H under reducing conditions, and (*b*) the content of cystine in the cysteine was negligible.

Action of Hydrogen Sulfide on *l*-Cystine Solution.—A solution (0.001 *M*) of *l*-cystine in 0.03 *N* hydrochloric acid was saturated with hydrogen sulfide at 25°. After being kept (stoppered) at this temperature for six hours, liberation of sulfur became noticeable; after twenty-eight and one-half hours and one hundred and seventy-one hours, the ultraviolet absorption spectrum was recorded (see Fig. 12). There was slow reduction of the cystine to cysteine, and after one hundred and seventy-one hours, the spectrum was practically identical with that of 0.002 *M* *l*-cysteine (in 0.03 *N* hydrochloric acid) saturated with hydrogen sulfide. [It has been stated (25) that hydrogen sulfide is incapable of reducing cystine to cysteine.]

SUMMARY

1 *l*-Cysteine, glutathione, and L-ascorbic acid have no reducing effect on alloxan in aqueous solution at *p*H 1.6.

2. At *p*H 1.6, there was no evidence of combination of alloxan with glutathione to give a substance whose ultraviolet absorption spectrum exhibited a peak at 305 mμ.

3. At *p*H 1.6, *l*-cysteine affords no appreciable protective action against aerial oxidation of alloxantin solution; hence, at this *p*H, dialuric

acid is a stronger reducing agent than *l*-cysteine. It is, however, a weaker reductant than hydrogen sulfide.

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Some Resistance Studies on a Smooth Phase and a Flocculent Variant of *Pseudomonas aeruginosa**

By C. GAINOR and C. K. WILLIAMSON

The bactericidal action of heat, iodine, and streptomycin on two forms of *Pseudomonas aeruginosa* are reported. The flocculent variant of the organism was more resistant to heat and iodine, but less toxic for mice and less resistant to streptomycin, than the smooth phase.

THE numerous reports concerning forms of bacteria resistant to drug action have indicated a need for more specific information correlating recognizable bacterial characteristics with relative susceptibility of the organisms to physical and chemical agents. This information is particularly important in connection with the present practice of using as test organisms certain bacteria with known standard susceptibilities to antibiotics. Dissociation of the particular organisms has necessitated considerable additional work (1), and indicates that misleading evaluations of drug potency may possibly result if changes in bacterial characteristics are not detected.

In a recent publication by McLean, *et al.* (2), the data indicated that chloramphenicol was more effective against a smooth type of *Pseudomonas aeruginosa* isolated from a patient than against a rough type of this organism obtained from the same patient. Schnitzer, *et al.* (3), reported that colonial differences in a strain of *Micrococcus pyogenes* var. *albus* could be correlated to differences in resistance to penicillin.

The literature contains several reports indicating that many pathogenic bacteria may exist in different forms in the body during natural infection and convalescence (4, 5), as well as in experimental *in vivo* studies (6). In the studies cited above, the rough form of the respective organisms has been observed.

Our choice of organism was stimulated by the comprehensive review by Stanley (7), dealing with numerous recorded studies of *Pseudomonas aeruginosa*. These studies concerned cases in which the organism had been associated with such diverse pathological conditions as genitourinary, endocarditic, and blood-stream infections.

EXPERIMENTAL

Culture Studies.—Viewed by transmitted light, the colonies of the original culture of *Pseudomonas aeruginosa* (A.T.C.C. 10,145) had a raised, opaque, glistening center bordered by a less opaque, dull, depressed, concentric ring. The colony mass

* Received April 9, 1951, from the Division of Bacteriology, Department of Biological Sciences, University of Pittsburgh.

tapered gradually from the outer edge of this ring to an irregular translucent margin. The smooth phase was obtained by culturing the original strain in Difco nutrient broth containing 1% LiCl. After two twenty-four-hour subtransfers in this broth, the resulting colonies on agar were convex, entire, opaque, glistening, homogeneous in 0.85% saline, and produced pyocyanin in good quantity. The flocculent variant was obtained by culturing the original strain in increasing concentrations of its own products of metabolism. The broth culture was Seitz-filtered and reinoculated at eight-day intervals three successive times. The flocculent variant was nonpyocyanogenic and clumped spontaneously in 0.85% saline. The colonies had a raised, opaque, bead-like center and an irregular, lobate, translucent periphery. The surface was dull and finely wrinkled. It should be pointed out that this was not a true rough-phase culture, although similar morphological forms have been cited as such in the literature (8).

The smooth phase was maintained on Difco tryptone-glucose-extract agar, and the flocculent variant was cultured on Difco nutrient agar. These media could not be used interchangeably without effecting alterations in the colonial morphology. However, the respective forms were stable throughout the course of repeated subtransfers on the original solid media. In all of the resistance studies, the flocculent cells, suspended in 0.85% saline adjusted to *p*H 7.0, were rendered to a smooth homogeneity by physical bombardment with glass beads in a 125-ml. Erlenmeyer flask. This was necessary to insure a uniform inoculum of cells in each exposure to heat and chemicals. The smooth cells were treated similarly so that conditions were kept constant. Prior to subjecting both types of cells to either chemical or physical agents, the cell suspensions were diluted with sterile distilled water to the desired turbidity. Eighteen-hour agar-slant cultures were used in all of the studies presented. All resistance studies were done in quadruplicate.

Streptomycin Studies.—Streptomycin Merck (calcium chloride complex) was prepared by dissolving 1 Gm. of streptomycin base with 9.0 ml. of sterile distilled water. This stock solution, containing 100 mg./ml. of active streptomycin, was further diluted with sterile distilled water to give concentrations of 100, 200, and 300 μg./ml. In this and subsequent experiments, the diluting menstruum used was sterile distilled water, rather than so-called physiological saline, to avoid introducing the possible additive factor of toxicity due to the salt (9). One milliliter of an estimated number of cells of the smooth phase or of the flocculent variant was exposed to the above concentrations of streptomycin for one, five, ten, and thirty minutes. In this manner, both exposure time and concentration of the antibiotic were varied. After each exposure time, 1 ml. of the suspension was transferred to 99-ml. dilution blanks of sterile distilled water to nullify action of the streptomycin. One milliliter was then transferred to a Petri dish, and plates were poured for total counts in the usual manner. In preliminary studies, broth cultures were made concomitantly to check for possible bacteriostasis, but these were discontinued because the results paralleled the quantitative studies. The results of the streptomycin studies are presented in Table I. The smooth-phase culture of *Pseudomonas aeruginosa* was considerably more resistant than the flocculent variant to the effects of streptomycin. This greater resistance was substantiated by the filter-disk assay method.

Iodine Studies.—The aqueous iodine was prepared according to the modified formula for Lugol's solution (U. S. P. XIV), and the 0.001, 0.0005, and 0.0001% dilutions were made with sterile distilled water. The experimental procedure was the same as that presented under Streptomycin Studies, except that 9-ml. dilution blanks of 0.1 *N* sodium thiosulfate were used to neutralize the aqueous iodine. The flocculent variant of *Pseudomonas aeruginosa* was more than five times as resistant as the smooth

TABLE I.—A COMPARISON OF THE RELATIVE RESISTANCE TO STREPTOMYCIN OF A SMOOTH PHASE AND A FLOCCULENT VARIANT OF PSEUDOMONAS AERUGINOSA

Streptomycin, μg./Ml.	Smooth Phase at 37°				Flocculent Variant at 37°			
	Exposure Time, Min.				Exposure Time, Min.			
	1	5	10	30	1	5	10	30
100	75×10^{4a}	27×10^{4}	50×10^{4}	40×10^{4}	90×10^{3}	80×10^{3}	0	0
200	85×10^{4}	34×10^{3}	42×10^{3}	28×10^{3}	90×10^{3}	180	0	0
300	30×10^{4}	24×10^{3}	10×10^{3}	18×10^{3}	90	0	0	0
Control	33×10^{5}	[b]	....	40×10^{5}	13×10^{5}	....	.	20×10^{5}

[a] Number of colonies surviving per ml.
[b] No determination made.

TABLE II.—A COMPARISON OF THE RELATIVE RESISTANCE TO AQUEOUS IODINE OF A SMOOTH PHASE AND FLOCCULENT VARIANT OF PSEUDOMONAS AERUGINOSA

Aqueous Iodine,[a] %	Smooth Phase at 37°				Flocculent Variant at 37°			
	Exposure Time, Min.				Exposure Time, Min.			
	1	5	10	30	1	5	10	30
0.001	0[b]	0	0	0	40	0	0	0
0.0005	45×10^{3}	0	0	0	440	120	120	0
0.0001	13×10^{5}	74×10^{4}	14×10^{4}	200	22×10^{4}	11×10^{4}	12×10^{4}	12×10^{3}
Control	24×10^{5}	[c]	..	12×10^{5}	17×10^{4}	..	..	15×10^{5}

[a] 1 Gm. of iodine (C. P.) + 2 Gm. KI in 100 ml. of H_2O = 1% aqueous iodine.
[b] Number of colonies surviving per ml.
[c] No determinations made.

TABLE III.—A COMPARISON OF THE RELATIVE RESISTANCE TO HEAT AT 58° OF A SMOOTH PHASE AND FLOCCULENT VARIANT OF PSEUDOMONAS AERUGINOSA

	Smooth Phase							Flocculent Variant						
	Exposure Time, Min.							Exposure Time, Min.						
Heat	0	1	5	10	15	20	30	0	1	5	10	15	20	30
	42×10^4	18^a	4	0	...[b]	0	0	4×10^4	19×10^3	22×10^3	60	...	4	0
Control[c]	42×10^4	..	.	.	11×10^4	.	16×10^4	4×10^4	..	..	..	2×10	.	4×10^4

[a] Number of colonies surviving per ml. [b] No determinations made. [c] Control at 37°.

phase to the effects of aqueous iodine (Table II). It should be noted that the initial cell concentration of the flocculent variant was less than that of the smooth phase.

Heat Studies.—The cells of the smooth phase and the flocculent variant were suspended in 20 ml. of sterile distilled water, *p*H 7.0, in 50-ml. test tubes. The tubes were immersed in a thermostatically controlled constant-temperature water bath regulated at 58°. The time for an equal volume of sterile distilled water to be raised to 58° was determined. The tubes containing the test organisms were subjected to the same procedure. When the desired temperature was reached inside the tubes, 1-ml. aliquots of the respective cell suspensions were taken at zero, five-, ten-, twenty-, and thirty-minute intervals. This procedure was followed in order to avoid sudden temperature shock to the cells. These portions were plated directly and in serial dilutions of 1:100 and 1:10,000. Plates were poured for total counts in the usual manner. The flocculent variant of *Pseudomonas aeruginosa* was four times as resistant as the smooth phase to the effect of temperature (Table III).

Toxicity Studies.—In a preliminary study, 36 mice, each weighing approximately 30 Gm., were inoculated intraperitoneally with 0.5 ml. of an eighteen-hour suspension of cells in various concentrations. Three groups of six mice each received inoculations containing 25×10^6, 25×10^4, and 25×10^3 cells, respectively, of smooth-phase organisms; three other groups of six mice each received inoculations containing 80×10^6, 80×10^4, and 80×10^3 cells, respectively, of the flocculent variant. Four of the six mice inoculated with 25×10^6 smooth-phase cells died within twenty-four hours, whereas two of the six mice receiving 80×10^6 flocculent cells died in the same period. A third mouse from the latter group died in the twenty-eighth hour. The organisms were recovered from the heart blood in each of the dead mice. All other mice survived for a week. It should be noted that although both types of cells were toxic for the mice, the smooth-phase organisms exerted a greater effect than did the flocculent type, especially since the cell concentration in the smooth-phase inoculum was less than one-third that in the flocculent inoculum.

DISCUSSION

The two most important findings in this study are that recognizable bacterial characteristics can be correlated with resistance to germicidal agents, and that the degree of resistance may differ with the agent employed.

Our results are in theoretical agreement with those of Torrey and Montu (10), who found that a rough variant of *Escherichia coli* exhibited greater resistance to heat than a smooth phase of the same culture. Our findings concerning the greater resistance of our flocculent variant both to heat and to iodine also parallel the results obtained by Davis, *et al.* (11). They indicated that spores of *Bacillus globigii* displaying relatively great resistance to heat showed the same tendency toward iodine, whereas these spores and spores of the parent organism displaying less resistance to heat and iodine showed equal susceptibility to streptomycin. These authors did not mention differences in bacterial characteristics of the two types of spores. Moreover, the efficiency of the 0.0001% aqueous iodine against the smooth phase of *Pseudomonas aeruginosa* is comparable to the performance observed by Beckwith and Moser (12), of an iodine tincture of the same concentration against *Escherichia coli*. The greater resistance of our flocculent variant is apparent.

The slightly greater virulence for mice of our smooth-phase organisms conforms to the general concept concerning the relationship of colonial phases to pathogenicity (13).

Although the data presented in Tables I to III are the results of single comparative tests, all similar trials yielded corroborative values.

SUMMARY

A smooth-phase culture of *Pseudomonas aeruginosa* displayed greater virulence for mice and greater resistance to streptomycin than did a flocculent variant of the same organism.

The flocculent variant was more resistant to heat and iodine.

It is suggested that resistance of bacteria to physical and chemical agents be correlated to morphological and biological characteristics of the bacteria.

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A Study of the Toxicity and Local Anesthetic Properties of Some Derivatives of Cumic Acid*,†

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Three dialkylaminoalkanol esters of cumic acid (*p*-isopropyl benzoic) have been investigated pharmacologically for acute toxicity, irritant action, and local anesthetic properties. Results obtained indicated that β-diethylaminoethyl cumate hydrochloride showed activity equal to procaine hydrochloride. It produced a slight fall in blood pressure and increase in respiration on intravenous injection. No prolonged effects were seen from doses as high as 4 cc. of 3 per cent. Duration of anesthesia was increased considerably by epinephrine. The other two compounds were effective but inferior.

THREE dialkylaminoalkanol esters of cumic acid in the form of their hydrochlorides were investigated pharmacologically in an attempt to evaluate their desirability for local anesthetic use. Determinations of acute toxicity, irritation, effects on blood pressure and respiration, and various phases of local anesthetic activity were made. All three compounds were found to be effective in local anesthetic tests and the β-diethylaminoethyl ester hydrochloride, on the basis of toxicity and anesthetic potency, compared favorably with procaine hydrochloride.

The synthesis of derivatives of cumic (*para* isopropyl benzoic) acid has been the subject of several studies conducted at this laboratory. Carkhuff and Gramling in 1948 (1) synthesized several aliphatic and aromatic esters; Baxter and Foote (2) produced nitro derivatives; and Bryan and Foote in 1950 (3), with the idea of producing derivatives with local anesthetic activity, synthesized the following dialkylaminoalkanol esters: β-dimethylaminoethyl cumate, β-diethylaminoethyl cumate, and γ-diethylaminopropyl cumate. The similarity of the structures of these compounds to that of procaine suggests local anesthetic properties, and preliminary tests by Bryan and Foote (3) confirmed this. The evaluation of the various phases of this local anesthetic activity and investigation of the toxicity and irritant properties of these three compounds was the object of this study. The hydrochlorides of the esters were used since the free esters are not water soluble.

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ACUTE TOXICITY

Median lethal doses of the three compounds were determined in white Swiss mice, random sex sampling, by intraperitoneal injection. The median lethal dose of procaine hydrochloride determined in the same manner as that employed for the cumic acid esters was used for comparison. The interpretation of the data for estimation of the LD_{50} was done according to the system of Miller and Tainter (4). The results are presented in Table I.

Symptoms observed were typical of local anesthetic toxicity and were related to central nervous system stimulation. Excitement, erratic movements, convulsions which were predominantly clonic, and respiratory difficulties were seen. Death was evidently due to exhaustion and respiratory depression following excessive stimulation. A period of lethargy following the convulsions usually preceded death. Following injection of procaine and the γ-diethylaminopropyl compound a more rapid onset of action was observed, with death following more quickly than with the other compounds. There were no gross indications of damage on examination at necropsy.

IRRITATION

The three compounds were tested for irritant action by subcutaneous injection into the backs of rabbits. Hair was removed from the backs of the rabbits by use of a depilatory of equal parts of acacia and barium sulfide, made to a paste with water. Following removal of hair, further procedure was delayed twenty-four hours to permit recovery from the slight irritation of the depilatory. At the time of injection the skin appeared normal and free from irritation. Fifteen rabbits were used in testing, five for each compound. Three concentrations, 0.5, 3, and 5%, all in 0.5-cc. amounts, were injected subcutaneously into different areas of the back of each rabbit, and the sites were marked with ink. The results of the injections were noted at intervals during forty-eight hours. Results are presented in Table II. In rating degree of irritation the area of involvement, erythema, necrosis, induration, edema, and general appearance were considered.

From Table II it is evident that only slight irritation may be expected from use of β-diethylaminoethyl cumate hydrochloride in solution of 3%

TABLE I.—ACUTE TOXICITY: INTRAPERITONEAL WHITE SWISS MICE

Compound	Dose, Mg./Kg.	Mortality 12 Hr.	Mortality 36 Hr.	LD50, Mg./Kg.
β-Diethylaminoethyl cumate HCl	200	0/10	0/10	370 ±18.2
	300	2/10	2/10	
	350	0/10	4/10	
	400	4/10	7/10	
	450	4/10	7/10	
	500	8/10	9/10	
	600	10/10	10/10	
β-Dimethylaminoethyl	350	4/10	4/10	376 ±18.8
	400	3/10	5/10	
	450	9/10	9/10	
	500	8/10	10/10	
γ-Diethylaminopropyl cumate HCl	200	0/10	0/10	238 ±15.5
	225	4/10	4/10	
	250	6/10	6/10	
	275	4/10	7/10	
	300	10/10	10/10	
	350	10/10	10/10	
Procaine HCl	200	0/10	0/10	275 ±12.6
	250	3/10	3/10	
	275	5/10	5/10	
	300	7/10	7/10	
	400	10/10	10/10	

TABLE II.—IRRITATION BY SUBCUTANEOUS INJECTION IN RABBITS

Compound	0.5%, 0.5 Cc.	3%, 0.5 Cc.	5%, 0.5 Cc.
β-Diethylaminoethyl cumate HCl	0*	0	0
	0	0	0
	0	0	+
	0	+	++
	0	0	++
β-Dimethylaminoethyl cumate HCl	0	++	++
	0	+	+
	0	++	++
	0	+	++
	0	0	+
γ-Diethylaminopropyl cumate HCl	0	++	++
	0	++	+++
	0	+	++
	0	+	+
	0	+	+

* 0 = Nonirritating, + = slight irritation, etc.

strength. The 5% solution appears to be altogether too irritant for general use. Solutions of 0.5% of β-dimethylaminoethyl cumate hydrochloride apparently cause no irritation. Solutions of 3% showed definite signs of irritation and indicated they should be used with caution. The γ-diethylaminopropyl compound appeared slightly more irritant, only the 0.5% solution appearing to be free of irritant action.

LOCAL ANESTHESIA

Topical Anesthesia.—Abolition of the corneal reflex in rabbits, a method similar to that described by Sollmann (5) was used in determining topical anesthetic potency. Preliminary screening tests showed that all three compounds were effective in abolishing the reflex but were considerably inferior to cocaine hydrochloride, which was used as a control. γ-Diethylaminopropyl cumate hydrochloride, the most effective of the three, was tested further by the same method. The cumic ester was applied to the left eye; cocaine hydrochloride to the right, both in a concentration of 1.5% in normal saline. Mean durations of anesthesia in minutes for the 10 rabbits used were:

γ-Diethylaminopropyl cumate hydrochloride	16.1 ± 1.9
Cocaine hydrochloride	33.5 ± 3.1

Irritation from the γ-diethylaminopropyl cumate hydrochloride application, as revealed by erythema of the cornea and conjunctiva, was observed in six of the rabbits.

Nerve Block.—The effectiveness of the three compounds in producing nerve block was determined on frogs using the lumbar plexus method of Bülbring and Wajda (6). Procaine hydrochloride was used as a control. Each compound was used in a concentration of 0.1% in 0.7% saline. Results expressed as time required for onset of action are presented in Table III. The compound which proved most effective in this test, β-diethylaminoethyl cumate hydrochloride, was the subject of further testing by comparison with procaine in ability to produce nerve block in the sciatic nerve of the rabbit. The method of Schmitz and Loevenhart was used (7). In experiments on four rabbits, the cumate compound applied to the right sciatic, procaine to the left, there was abolition of the respiratory response by both compounds in 0.5% solution, the 0.1 and 0.3% solutions proving ineffective in both instances.

Infiltration Anesthesia.—Anesthetic activity following infiltration of each of the three compounds was evaluated by the guinea-pig wheal method of Bülbring and Wajda (6). Preliminary tests showed that a 0.1% solution was effective in producing anesthesia, and the three cumate compounds and procaine used as a control were employed at this concentration in 0.25-cc. injections. Results, expressed as the means of failure of response to pin pricks during the thirty-minute testing period, are presented

TABLE III.—TIME OF ONSET OF FROG LUMBAR PLEXUS ANESTHESIA, IN MINUTES

	β-Diethylaminoethyl Cumate HCl, 0.1%	β-Dimethylaminoethyl Cumate HCl, 0.1%	γ-Diethylaminopropyl Cumate HCl, 0.1%	Procaine HCl, 0.1%
	3.5	4.5	6.5	4
	2.5	5	7	3.5
	3	3	3	3
	3	7	2	3
	3.5	4	4.5	5
	2	1	1.5	3.5
	1.5	2	1.5	3
	4	3	3	1
	1.5	3.5	3.5	4
	2.5	4	3.5	3
Mean	2.7	3.7	3.6	3.3
Std. Error	±0.27	±0.52	±0.6	±0.33

TABLE IV.—INTRADERMAL ANESTHESIA IN GUINEA PIGS

Compound	No. of Times No Response to Pin Pricks									
	G. P. I.		G. P. II		G. P. III		G. P. IV		G. P. V.	
	Front	Back	Front	Back	Front	Back	Front	Back	Front	Back
Procaine HCl, 0.25 cc., 0.1%	20	25	18	20	7	11	14	22	7	12
β-Diethylaminoethyl cumate HCl, 0.25 cc., 0.1%	14	30	8	21	9	30	11	18	16	19
β-Dimethylaminoethyl cumate HCl, 0.25 cc., 0.1%	3	4	4	10	2	9	2	6	2	9
γ-Diethylaminopropyl cumate HCl, 0.25 cc., 0.1%	9	12	8	22	13	15	9	20	16	17

in Table IV. They indicate that the β-diethylaminoethyl and the γ-diethylaminopropyl compounds are approximately equal to procaine in producing this type of anesthesia. The only sign of irritation from these injections was a slight and transient erythema following injections of the γ-diethylaminopropyl derivative. This was observed in only four of the ten injections of this compound.

Since, on the basis of the toxicity, irritation, and local anesthesia tests, the β-diethylaminoethyl compound proved to be the most desirable of the three for local anesthetic use, further testing was confined to it.

Another test of infiltration anesthesia was devised which was based on the ability of the subcutaneously injected anesthetic to abolish the wink reflex response to touching the lower eyelid of the rabbit. The eyelashes were clipped and the normal response was tested by touching the lower lid with the point of a pencil. The anesthetic solution was injected subcutaneously into the lower lid just below the point of emergence of the cut eyelashes. One cubic centimeter of 0.3% solutions of β-diethylaminoethyl cumate hydrochloride and procaine hydrochloride were injected into both eyes of each rabbit, each solution given in two 0.5-cc. injections approximately 1 cm. apart. Since anesthesia was immediate in both cases, only duration of anesthesia was recorded. Ten rabbits were used in the test. The results are presented in Table V and show the two compounds to be of approximately equal anesthetic activity. To test the validity of this method, injections of the solvent (distilled water) were made in the same manner. Two injections of 0.5 cc. each injected into the lower eyelid caused only a light anesthesia for a short time. Injections of 0.25 cc. produced no anesthesia in any of the animals tested, whereas injections of procaine hydrochloride in 0.1% solution in both these amounts produced definite anesthesia for from fifteen to thirty minutes.

Potentiation by Epinephrine.—Experiments were performed to determine the effect of epinephrine when used in conjunction with the anesthetic. It was found that anesthesia is prolonged by epinephrine. Without epinephrine, as tested by injections of 0.1% solutions of the drug into the lower eyelids of seven rabbits, the mean duration of anesthesia was ten minutes. After addition of 1:40,000 solution of epinephrine, the same concentration produced local anesthesia which had a mean duration of fifty-three minutes.

Spinal Anesthesia.—Spinal anesthesia was produced in three dogs using β-diethylaminoethyl cumate hydrochloride, and in one dog using procaine hydrochloride. Aseptic technique was employed in all except one dog, in which case the solution of the

TABLE V.—DURATION OF INFILTRATION ANESTHESIA IN LOWER EYELID OF RABBIT

Rabbit	β-Dimethylaminoethyl Cumate HCl, 1 cc., 0.3%	Procaine HCl, 1 cc., 0.3%
I	19	25
II	24	17
III	20	20
IV	23	17
V	26	25
VI	20	23
VII	22	22
VIII	1 hr. 40 min.*	33
IX	26	18
X	40	16
Mean	24	23
Std. Error	±2	±1.7

* Discarded.

TABLE VI.—SPINAL ANESTHESIA IN DOGS

Dog	Weight, Kg.	Anesthetic	Dose	Duration
I	7.5	Procaine HCl	1/2 cc., 4%	1 hr. 10 min.
II	4	β-Diethylaminoethyl cumate HCl	1/2 cc., 4%	1 hr. 32 min.
III	6	β-Diethylaminoethyl cumate HCl	1/2 cc., 4%	40 min.
IV	6	β-Diethylaminoethyl cumate HCl	1/2 cc., 4%	50 min.

cumate ester hydrochloride was left unautoclaved to determine any loss of activity which might be due to hydrolytic destruction as a result of autoclaving.

Injections according to standard procedure were made into the subarachnoid space at a point between the fifth lumbar and first sacral vertebrae. Withdrawal of spinal fluid confirmed the correct position of the needle. Following injection, the dogs were held in position for several minutes. At intervals the skin in the hind quarter of the body was pinched with toothed forceps to determine the beginning and end of anesthesia. The results of these injections are given in Table VI, from which it is evident that β-diethylaminomethyl cumate hydrochloride is effective in producing spinal anesthesia. Motor nerves were also affected. The onset of this action was not as rapid as the sensory nerve action; however, in all cases there was complete loss of the motor function in the area involved, evidenced by the inability to use the hind legs in walking. The duration of this action was slightly less than the sensory effect in dogs I, II, and III. In dog IV there was persistence of motor paralysis after return of response to sensory stimuli had begun. The effectiveness of the compound was apparently affected only slightly if at all by autoclaving. Although one procaine control is not sufficient basis for a statistical conclusion, the indication is that the cumate compound is slightly less effective than procaine in producing spinal anesthesia. The dogs were observed for two weeks following injection. Dog II injected with the cumate compound showed a slight stiffness in the hind quarter for forty-eight hours. None of the other dogs showed any symptoms of damage.

BLOOD PRESSURE AND RESPIRATION

The effects of β-diethylaminoethyl cumate hydrochloride on blood pressure and respiration were determined in experiments on three dogs by cannulation of the tracheae and left carotid arteries. Injections were made through cannulated femoral veins.

There were no significant changes in blood pressure on injection of a 1% solution in doses as high as 4 cc. The only effect on respiration was a slight and momentary respiratory apnea.

Effects from injection of the 3% solution were of short duration and of no great magnitude. Following 1-cc. doses there was a slight rise, followed by a greater fall in blood pressure. Pulse pressure increased as pressure returned to normal. Respiration changes consisted of a momentary apnea followed by a slight increase in rate and amplitude. Doses of 4 cc. caused the same type of blood pressure response, with a more predominant fall in pressure after the preliminary rise. Respiration was increased in amplitude and rate. These changes were of short duration and were followed by complete return to normal as Fig. 1 shows. Effects of the cumate compound were similar in pattern and magnitude to those following intravenous injection of the same doses of procaine hydrochloride.

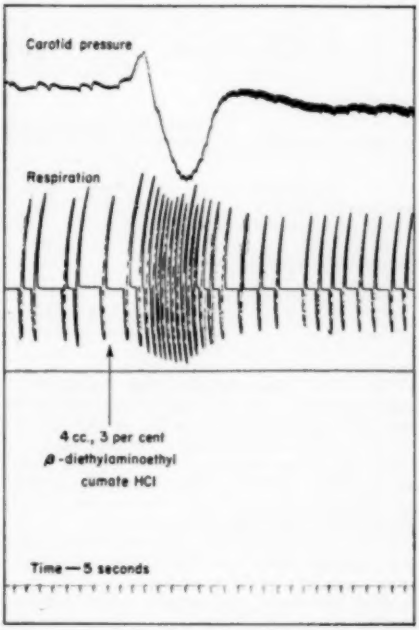

Figure 1

SUMMARY

Three dialkylaminoalkanol esters of cumic acid in the form of the hydrochlorides have been investigated pharmacologically in an attempt to evaluate their practicality for use as local anesthetic agents.

On the basis of toxicity, irritation, local anesthesia tests, and blood pressure and respiration determinations, β-diethylaminoethyl cumate hydrochloride compared favorably with procaine hydrochloride.

The other two derivatives, β-dimethylaminoethyl cumate hydrochloride and γ-diethylaminopropyl cumate hydrochloride, were effective in local anesthetic tests, but proved less desirable on the basis of toxicity, irritation, and degree of anesthesia produced.

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A Comparative Study of the Biological and Chemical Assays of Aureomycin Treated Under Certain Conditions*

By S. W. GOLDSTEIN,† D. C. GROVE,‡ W. W. WRIGHT,‡ A. KIRSHBAUM,‡ and H. R. HERNANDEZ†

When aureomycin hydrochloride is heated in the presence of water, the bioassay shows a lower potency value than is indicated by physicochemical assay methods. Hydrolytic degradation products of aureomycin hydrochloride influence the physicochemical assay results, but products resulting from thermal degradation of dry aureomycin hydrochloride do not interfere with the chemical assays. Physicochemical assay methods can be substituted for the bioassay when a comparison of the two procedures has shown agreement. The potency of aureomycin hydrochloride in glycerinated gelatin suppositories prepared according to the U. S. P. procedure can be determined only by the bioassay method. The effects of different treatments upon the ultraviolet spectrum of aureomycin are reported.

THIS STUDY was undertaken to determine the stability of aureomycin during the manufacture and the storage of glycerinated gelatin suppositories of aureomycin hydrochloride.

Bioassay and physicochemical assay methods were used to determine the active concentration of aureomycin. The turbidimetric method (1) utilizing *M. pyogenes* var. *aureus* (P. C. I. 209-P and American Type Culture Collection 6538-P) was used for the bioassays. Two physicochemical methods were employed: (*a*) A colorimetric method based on the spectrophotometric measurement of the yellow color developed when aureomycin is heated with acid (2). (*b*) A direct spectral method based on the measurement of the light transmitted by aqueous acid solutions of aureomycin hydrochloride at 369 mμ (3). Data obtained with solutions of aureomycin hydrochloride at wave length 369 mμ show that the values obtained are in accord with Beer's law (Fig. 1).

Results obtained on aureomycin suppositories by the bioassay method showed a lower potency than was indicated by the physicochemical methods. This led to further study of these methods when applied to aureomycin hydrochloride that had been treated under different conditions, in order to ascertain whether the chemical methods measure degradation products in addition to active aureomycin.

EXPERIMENTAL

Two batches of aureomycin glycerinated gelatin suppositories were prepared to contain 105 mg. of aureomycin hydrochloride per suppository as follows: the weighed quantity of aureomycin hydrochloride[1] was suspended in a small quantity of warm

* Received July 6, 1951, from the Laboratory of the AMERICAN PHARMACEUTICAL ASSOCIATION and the Federal Security Agency, Food and Drug Administration, Division of Antibiotics, Washington, D. C.
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‡ Federal Security Agency, Food and Drug Administration, Division of Antibiotics.

[1] Biological assay 96%, colorimetric assay 104%, and direct spectral assay 101%. The aureomycin hydrochloride was obtained through the courtesy of Lederle Laboratories Division, American Cyanamid Co.

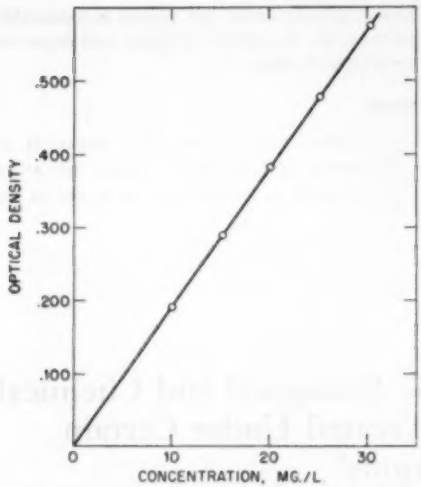

Fig. 1.—Standard curve showing the relation of aureomycin hydrochloride concentration in 0.1 *N* HCl to optical density at 369 mμ.

glycerin. This suspension was then transferred to a sufficient quantity of melted U. S. P. glycerinated gelatin base (on a bath at 100°). The thoroughly mixed mass was then poured into molds and refrigerated. The addition, mixing, and pouring into molds required maintenance at about 100° for approximately ten to fifteen minutes. The suppositories were prepared for assay as follows: an individual suppository and 200 cc. of distilled water were placed in a Waring blendor jar and blended for five to ten minutes. Aliquots of this stock solution were further diluted and assayed by the biological and chemical methods previously mentioned.

The suppositories were stored in a refrigerator and samples were taken for assay at various intervals. The results obtained are shown in Table I. Allowing for the possibility that individual suppository variations could be partly attributable to uneven drug distribution and to assay deviations, the results obtained show that: (*a*) The biological assay method gives values considerably lower than those obtained by either of the physicochemical methods. (*b*) The bioassay indicates that there is a further loss of potency during storage in the refrigerator. (*c*) The colorimetric assay method tends to give slightly higher results than the direct spectral measurement method at 369 mμ.

Because the biological assay showed a loss of about 33% of potency on Lot 1 on the same day the suppositories were prepared, it was suspected that this loss of activity was due to degradation of aureomycin by heat and moisture present during preparation. In order to eliminate the possibility that the glycerin or gelatin might have some effect on the growth of the organism used in the biological assay, a blank glycerinated gelatin suppository containing no aureomycin was assayed and showed no effect on the organism. Standard aureomycin when added to the stock solution of the blank suppository gave 100% recovery by the bioassay method. The solution of the blank suppository showed no absorption at 369 mμ and also gave no interference in the colorimetric assay.

TABLE I.—BIOLOGICAL AND CHEMICAL ASSAY DATA FOR AUREOMYCIN GLYCERINATED GELATIN SUPPOSITORIES STORED IN REFRIGERATOR

Lot No.	Time of Storage, Days	Bioassay Method, Mg./Supp.	Direct Spectral Method, Mg./Supp.	Colorimetric Method, Mg./Supp.
1	0	67.1	91.0	99.0
	1	66.0	85.3	97.2
	2	62.7	95.0	98.2
	3	60.0	104.0	103.8
	5	67.2	93.1	...
	12	57.6	98.0	...
	23	54.0	103.4	...
	30	40.0	69.8	...
	36	50.0	95.8	...
	43	53.4	89.6	98.3
	61	43.9	90.6	94.9
2	0	...	99.2	100.8
	1	...	101.3	108.8
	2	...	103.8	109.5
	3	...	104.3	110.0
	10	...	102.4	107.5
	17	...	100.0	109.7
	27	...	101.5	109.6
	34	...	99.5	106.5
	43	...	99.0	106.7
	44	60.0	98.2	100.7

EFFECT OF VARIOUS TREATMENTS UPON AUREOMYCIN

Portions of dry aureomycin hydrochloride powder were oven-heated at 100° for periods of fifteen minutes to three hours, and at 200° for thirty to ninety minutes. The heating at 200° for ninety minutes caused a discoloration of the powder. The results which are given in Table II show that the dry powder is stable at 100° and is slowly decomposed at 200°. Essentially the same potency value, however, was obtained on the decomposed material by all assay methods.

TABLE II.—EFFECT OF HEAT UPON DRY AUREOMYCIN HYDROCHLORIDE

Time in Oven	Bioassay Method, μg./Mg.	Direct Spectral Method, μg./Mg.	Colorimetric Method, μg./Mg.
15 min., 100°	...	1,011	988
30 min., 100°	990	997	936
30 min., 100°	...	1,000	1,001
90 min., 100°	1,000	990	943
90 min., 100°	...	1,006	1,017
3 hr., 100°	...	1,000	1,007
30 min., 200°	933	1,020	...
90 min., 200°	603	645	609

Aureomycin hydrochloride powder was dissolved in boiling water and the solution was kept at 100° for a total of fifteen minutes. No change was noted in the spectral curve maxima for aureomycin in water. No change was noted in the spectral curve maxima after keeping an aqueous solution of aureomycin hydrochloride (*p*H 3.3) at room temperature for two days. Spectral curves for 0.1 *N* acid solu-

TABLE III.—ABSORPTION MAXIMA OF AUREOMYCIN AFTER VARIOUS TREATMENTS[a]

Soln. No.		Absorption Maxima in mμ		
1	Aureomycin hydrochloride in water (pH 3.3)	230	274	369
2	Aureomycin hydrochloride in 0.1 *N* HCl	230	262.5	368
3	Soln. 1 heated at 100° for 15 minutes	230	274	369
4	Soln. 3 plus acid to 0.1 *N* in HCl	230	262.5	369
5	Soln. 1 with blank glyc. gel. suppository	None	274	368
6	Soln. 5 plus acid to 0.1 *N* in HCl	None	266	369
7	Soln. 5 heated at 100° for 15 minutes	None	274	365
8	Soln. 7 plus acid to 0.1 *N* in HCl	None	266	369
9	Soln. 1 after 2 days at room temperature	230	274	369
10	Soln. 9 plus acid to 0.1 *N* in HCl	230	266	369
11	Heated dry drug at 100° for 15 minutes then dissolved in distilled water	230	276	369
12	Soln. 11 plus acid to 0.1 *N* in HCl	230	268	369
13	Aq. soln. of compounded suppository No. 1	None	260	368
14	Aq. soln. of compounded suppository No. 2	None	268	369
15	Soln. 13 plus acid to 0.1 *N* in HCl	None	266	369.5
16	Soln. 14 plus acid to 0.1 *N* in HCl	None	266	369.5
17	Soln. 13 after 4 days at 9°	None	270	365
18	Soln. 17 plus acid to 0.1 *N* in HCl	None	258	369

[a] All the transmissions were measured in a Beckman DU Spectrophotometer using a hydrogen lamp and water as a blank. Where acid is indicated, it was added just before testing.

tions of the drug, tested fresh and after two days at room temperature, showed no appreciable change in shape. Addition of acid to 0.1 *N* after heating an aqueous solution of the drug for fifteen minutes at 100° did not alter the shape of the curve obtained with the unheated acid solution. Solutions prepared from a compounded glycerinated gelatin suppository of aureomycin hydrochloride, or from the drug dissolved in a solution of a blank suppository, completely lost the absorption maximum at 230 mμ (water or acid), and the maxima at 274 mμ (water), 262.5 mμ (acid), and 369 mμ (water) were shifted. However, the maximum observed at 369 mμ in 0.1 *N* hydrochloric acid solutions was not appreciably disturbed by any of the above conditions or added materials (see Table III).

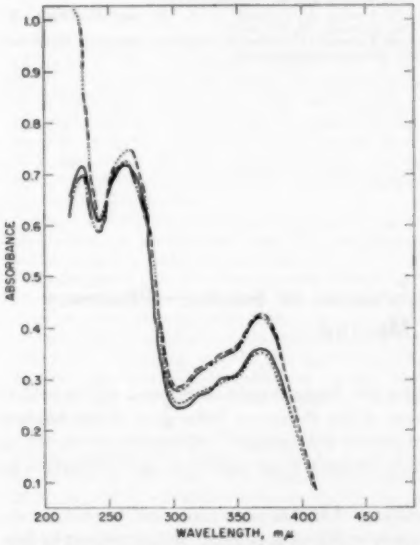

Fig. 2.—Aureomycin hydrochloride 20 μg./cc. in water. Readings were made after addition of HCl acid in each case.

Control ——·; control plus blank glycerinated gelatin suppository – – – –; control heated at 100° for fifteen minutes ———; control plus blank suppository heated at 100° for fifteen minutes · · · ·.

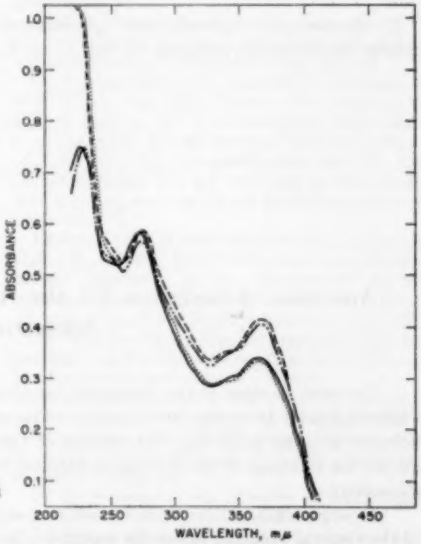

Fig. 3.—Aureomycin hydrochloride 20 μg./cc. in water.

Control (pH 3.3) – – – –; control plus blank glycerinated gelatin suppository (pH 4.3) ——— ———; control heated at 100° for fifteen minutes ———; control plus blank suppository heated at 100° for fifteen minutes · · · · · · · ·.

TABLE IV.—EFFECT OF WATER AT 100° UPON AUREOMYCIN HYDROCHLORIDE AS INDICATED BY BIOLOGICAL AND PHYSICOCHEMICAL ASSAY RESULTS

Time of Boiling, Min.	Bioassay Method, μg./Mg.	Direct Spectral Method, μg./Mg.	Colorimetric Method, μg./Mg.
10	827	919	959
10	806	939	918
15	812	965	963
15	849	989	997
30	700	840	...

Typical spectral curves for aureomycin in the ultraviolet band are given in Figs. 2 and 3. These curves also show the changes in absorbancy at the maximum at 369 mμ. The maximum at 369 mμ is not shifted by any treatment that aureomycin might be subjected to in the proper preparation of its usual dosage forms. When aureomycin has been altered by means of water, the spectral absorbance is lowered to an appreciably greater extent at 369 mμ than at the maxima at 230 and 274 mμ.

The effect of heat and water upon the potency as measured by the biological and physicochemical methods is shown in Table IV. These values were obtained by adding 1 Gm. of aureomycin hydrochloride powder to 20 cc. of boiling water and maintaining at 100°. It appears that when aureomycin is heated in water at 100° for periods up to thirty minutes, the bioassay shows a 12–15% greater loss in activity than is indicated by the physicochemical assay methods utilized.

SUMMARY

1. Aureomycin hydrochloride glycerinated gelatin suppositories prepared by the U. S. P. method show a loss of potency of the aureomycin when measured by the biological assay method. The two physicochemical methods used failed to measure this loss of potency and show that such methods would be unreliable for the assay of this preparation.

2. Aureomycin hydrochloride when heated in the dry state at 100° for ninety minutes showed no loss in potency by either the bioassay or physicochemical methods. When heated at 200° for ninety minutes, a loss in potency of approximately 40 per cent was obtained by both bioassay and chemical methods, indicating that the dry heat degradation products are not measured by the chemical methods.

3. When aureomycin hydrochloride is heated in the presence of water, the bioassay gives a lower potency value than the physicochemical methods, indicating that the latter methods measure some of the inactive hydrolytic degradation products in addition to active aureomycin.

4. Bioassay methods are the only reliable means of measuring the antibiotic activity of aureomycin. Physicochemical methods, however, may be substituted when a comparison of the two methods has shown agreement.

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American Association for the Advancement of Science—Pharmacy Subsection Meeting

The next meeting of the American Association for the Advancement of Science will be held in Philadelphia on December 26–31, 1951. The program of the Pharmacy Subsection of the Medical Sciences is being made up. Six sessions of this Subsection will be held. Members are invited to attend the meetings of the Section on Medical Sciences, Subsection on Medicine, and the Section on Chemistry.

Arrangements will be made for advance registration. Advance registrants will be sent a copy of the General Program before the meeting. See *Science* or *Scientific Monthly* in late August or September.

Authors who wish to present papers before the Subsection are requested to send in titles of papers *as soon as possible*. All titles for inclusion in the printed program must be in the hands of Glenn L. Jenkins, Chairman, Subsection Np, Purdue University, School of Pharmacy, Lafayette, Ind., by *September 15*.

Disinfection of Clinical Thermometers*

By LOUIS GERSHENFELD, ARTHUR GREENE, and BERNARD WITLIN

The antibacterial efficiency of each of several iodine solutions and different concentrations of ethyl alcohol and of isopropyl alcohol has been determined in connection with their use in the cold disinfection of clinical thermometers. Iodine tincture and iodine solution N. F. were found to be more effective than either of the alcohols for the quick disinfection of clinical thermometers infected with many of the commonly found bacteria.

A SEARCH of the literature on methods of disinfecting thermometers revealed only an occasional published study on the use of chemicals for the cold disinfection of clinical thermometers. It was therefore deemed advisable to study and evaluate the efficiency of some readily procurable compounds to be recommended for disinfecting thermometers. This study was conducted on the commonly used iodine preparations and various alcoholic solutions, compounds which are readily available, are economical, and are frequently employed as disinfectants.

EXPERIMENTAL

The compounds used were: (*a*) iodine solution (2%) N. F. IX; (*b*) iodine tincture (2%) U. S. P. XIV; (*c*) ethyl alcohol, 95%; (*d*) ethyl alcohol, 70% by weight (815 cc. of ethyl alcohol 95% at 25° and 1,000 cc. sterile distilled water); (*e*) ethyl alcohol, 50% by volume (45 cc. of ethyl alcohol 95% and 50 cc. of sterile distilled water); (*f*) isopropyl alcohol, 70% by volume; and (*g*) isopropyl alcohol, 50% by volume.

The tests were conducted on graduated clinical thermometers, which were cut into sections two inches long and sealed at both ends.

Technique.—(*a*) The thermometer pieces to be tested were washed with soap and hot water and then placed in chemically clean clay-top Petri dishes and sterilized in the hot air oven at 170° for one hour.

(*b*) The organisms used in this study were *Diplococcus pneumoniae, Staphylococcus aureus, Streptococcus hemolyticus, Streptococcus fecalis,* and *Escherichia coli.*

(*c*) Each test organism was transplanted at twenty-four-hour intervals in F. D. A. broth, prior to its use in the test. The clinical thermometer sections in the Petri dishes were covered by the culture of the test organism, so that 0.5 ml. of a twenty-four-hour broth culture was in the dish for each clinical thermometer section. The culture was swirled in the dish about the thermometer sections.

(*d*) The Petri dishes were placed in the incubator at 37° until the test organisms were thoroughly dried onto the thermometers.[1] This technique was followed except where otherwise indicated.

(*e*) The disinfectant to be tested was poured into a sterilized glass beaker (50-cc.) and the contaminated thermometer sections were transferred by sterile forceps from the Petri dish into the disinfectant solution. The thermometer sections remained in the disinfectant solution at 20° for a designated test period. They were then removed from the solution, rinsed for ten seconds in a beaker of sterile distilled water, and finally transferred to a tube of F. D. A. broth.[2] The transplant tubes were incubated for forty-eight hours at 37° and then examined for the presence or absence of growth. When iodine (tincture or solution) was used, 1% sodium thiosulfate was added to the broth as a halogen inactivator.

(*f*) It was deemed advisable to observe what effect organic matter, in addition to that already present in the culture, might have in its interference with the disinfecting action of the solution under test. For this purpose citrated human blood plasma was added to the culture-media tubes containing the twenty-four-hour growth of *S. aureus.* Citrated human blood plasma was added to the *S. aureus* culture tubes to obtain a total concentration of either 25 or 50%, and the contents of the tubes were mixed thoroughly.

(*g*) The plasma-culture mixture was poured over the thermometer sections in the Petri dish; 0.5 ml. was added for each thermometer section used.

(*h*) Control tests were conducted simultaneously with all of the experiments. The thermometer section used as the positive control was treated similarly to the method described under (*a*), (*b*), (*c*), (*d*), and (*e*), with the modification that the thermometer section was not immersed in the disinfectant solution, but was placed immediately into the broth tube.

Findings.—(*a*) It was observed that iodine solution (2%) killed *S. fecalis* following a one-hundred-second immersion of the thermometer section, whereas iodine tincture (2%) killed the organism after sixty seconds. Alcohol solutions, ethyl 50%, ethyl 70%, isopropyl 50%, and isopropyl 70%, were ineffective in preventing *S. fecalis* growth within one hundred and twenty seconds. (Table I.)

(*b*) All test solutions killed *S. hemolyticus* within a twenty-second immersion interval, with the exception of ethyl alcohol 95% which required eighty seconds to destroy this organism under conditions of the test. (Table II.)

(*c*) *D. pneumoniae* was killed by all test solutions within twenty seconds. (Table III.)

(*d*) Iodine solution (2%) and iodine tincture (2%) killed *E. coli* within one hundred seconds. Alcohol solutions, ethyl 70%, isopropyl 70%, and isopropyl

* Received June 7, 1951, from the Department of Bacteriology, Philadelphia College of Pharmacy and Science.

[1] *D. pneumoniae* and *S. hemolyticus* lysated when dried, and thus were left in the liquid state.

[2] In tests conducted with *D. pneumoniae* and *S. hemolyticus,* tryptic digest broth was used as the transplant medium.

50%, killed this test organism within one hundred and twenty seconds. Ethyl alcohol (95%) did not kill *E. coli* within one hundred and twenty seconds under conditions of the test. (Table IV.)

(*e*) Using *S. aureus* as the test organism, it was observed that iodine solution (2%) killed *S. aureus* within twenty-four seconds, whereas iodine tincture (2%) required only sixty seconds to kill the test organism. Isopropyl alcohol (70%) required three minutes to kill *S. aureus*. Alcohol solutions, ethyl 50% and 70%, and isopropyl alcohol 50%, killed *S. aureus* within five minutes. Ethyl alcohol 95% was ineffective as a disinfectant against *S. aureus* at all time periods employed. (Table V.)

(*f*) In tests conducted with a 50% citrated human plasma-culture mixture, using *S. aureus* as the test organism, it was observed that iodine solution (2%) killed *S. aureus* within five minutes. Iodine tincture (2%), ethyl alcohol 50% and 70%, and isopropyl alcohol 50% and 70% did not kill *S. aureus* following a ten-minute immersion of the contaminated thermometer sections in the above mixture. (Table VI.)

(*g*) In tests conducted with a 25% citrated human plasma-culture mixture, using *S. aureus* as the test organism, iodine solution (2%) killed the test organism within three minutes. Solutions of iodine tincture (2%), ethyl alcohol 50% and 70%, and isopropyl alcohol 50% and 70% did not kill *S. aureus* within ten minutes. (Table VII.)

SUMMARY

The antibacterial efficiency of various commonly used iodine solutions and different alcohols as agents for the cold disinfection of clinical thermometers was determined. The compounds used were iodine solution (2%) N. F. IX, iodine tincture (2%) U. S. P. XIV, ethyl alcohol (95%), ethyl alcohol (70% by weight), ethyl alcohol (50% by volume), isopropyl alcohol (70% by volume), and isopropyl alcohol (50% by volume).

Graduated clinical thermometers which were cut into sections two inches long and sealed at both ends were used. The clinical thermometer sections were

TABLE I.—RELATIVE EFFICIENCIES OF DISINFECTANT SOLUTIONS AGAINST S. FECALIS AFTER IMMERSION OF THERMOMETER SECTIONS

Immersion Time of Thermometer in Disinfectants, Sec.	Disinfectants Tested: Iod. Soln. 2% I_2	Iod. Tr. 2% I_2	Ethyl Alc. w/v 50%	Ethyl Alc. w/v 70%	Isop. Alc. w/v 70%	Isop. Alc. w/v 50%
Control Thermometer	+	+	+	+	+	+
20	+	+	+	+	+	+
40	+	0	+	+	+	+
60	+	0	+	+	+	+
80	+	0	+	+	+	+
100	0	0	+	+	+	+
120	0	0	+	+	+	+

+ = Growth after forty-eight hours.
0 = Growth after forty-eight hours.

TABLE II.—RELATIVE EFFICIENCIES OF DISINFECTANT SOLUTIONS AGAINST S. HEMOLYTICUS AFTER IMMERSION OF THERMOMETER SECTIONS

Immersion Time of Thermometer in Disinfectants, Sec.	Disinfectants Tested: Iod. Soln. 2% I_2	Iod. Tr. 2% I_2	Ethyl Alc. 95%	Ethyl Alc. 70%	Ethyl Alc. 50%	Isop. Alc. 70%	Isop. Alc. 50%
Control Thermometer	+	+	+	+	+	+	+
20	0	0	+	0	0	0	0
40	0	0	+	0	0	0	0
60	0	0	+	0	0	0	0
80	0	0	0	0	0	0	0
100	0	0	0	0	0	0	0
120	0	0	0	0	0	0	0

+ = Growth after forty-eight hours.
0 = Growth after forty-eight hours.

TABLE III.—RELATIVE EFFICIENCIES OF DISINFECTANT SOLUTIONS AGAINST D. PNEUMONIAE AFTER IMMERSION OF THERMOMETER SECTIONS

Immersion Time of Thermometer in Test Disinfectants, Sec.	Disinfectants Tested: Iod. Soln. 2% I_2	Iod. Tr. 2% I_2	Ethyl Alc. 95%	Ethyl Alc. 70%	Ethyl Alc. 50%	Isop. Alc. 70%	Isop. Alc. 50%
Control Thermometer	+	+	+	+	+	+	+
20	0	0	0	0	0	0	0
40	0	0	0	0	0	0	0
60	0	0	0	0	0	0	0
80	0	0	0	0	0	0	0
100	0	0	0	0	0	0	0
120	0	0	0	0	0	0	0

+ = Growth after forty-eight hours.
0 = Growth after forty-eight hours.

sterilized in the hot-air oven (one hour at 170°). The sterile sections were treated with cultures of test bacteria.

The test organisms used in the study were *Diplococcus pneumoniae, Staphylococcus aureus, Streptococcus hemolyticus, Streptococcus fecalis,* and *Escherichia coli.* Tryptic digest broth was used as the transplant medium for *S. hemolyticus* and *D. pneumoniae.* The other test organisms were transplanted into F. D. A. broth.

One-half milliliter of a twenty-four-hour culture of each test organism was placed separately in a Petri dish for each thermometer section. The Petri dishes were placed in the incubator at 37° until the test organisms thoroughly dried onto the thermometer sections. *D. pneumoniae* and *S. hemolyticus* were found to lysate when dried; accordingly, the sections were merely impregnated with the liquid cultures.

The disinfectant to be tested was poured into a sterilized glass beaker and the treated thermometer sections were aseptically transferred by forceps from the Petri dish into the disinfectant solution. The treated thermometer sections remained in the disinfectant solution at 20° for the designated time period, rinsed for ten seconds in sterile distilled water, and transferred to a tube of F. D. A. broth (*S. hemolyticus* and *D. pneumoniae* were transferred to tubes containing tryptic digest broth). Thermometer sections treated with iodine solutions were transferred after treatment to media containing 1% thiosulfate. The transplant tubes were incubated for forty-eight hours at 37°, and the cultures were examined for observation of growth.

In order to determine the effect of large quantities of organic matter upon the disinfectants under test, citrated human blood plasma in concentrations of 25 and 50%, was added to the initial culture

TABLE IV.—RELATIVE EFFICIENCIES OF DISINFECTANT SOLUTIONS AGAINST E. COLI AFTER IMMERSION OF THERMOMETER SECTIONS

Immersion Time of Thermometer in Test Disinfectants, Sec.	Disinfectants Tested						
	Iod. Soln. 2% I_2	Iod. Tr. 2% I_2	Ethyl Alc. 95%	Ethyl Alc. 70%	Ethyl Alc. 50%	Isop. Alc. 70%	Isop. Alc. 50%
Control Thermometer	+	+	+	+	+	+	+
20	+	+	+	+	+	+	+
40	+	+	+	+	+	+	+
60	+	+	+	+	+	+	+
80	+	+	+	+	+	+	+
100	0	0	+	+	+	+	+
120	0	0	+	0	0	0	0

+ = Growth after forty-eight hours.
0 = Growth after forty-eight hours.

TABLE V.—RELATIVE EFFICIENCIES OF DISINFECTANT SOLUTIONS AGAINST S. AUREUS AFTER IMMERSION OF THERMOMETER SECTIONS

Immersion Time of Thermometer in Test Disinfectants	Disinfectants Tested						
	Iod. Soln. 2% I_2	Iod. Tr. 2% I_2	Ethyl Alc. 95%	Ethyl Alc. 70%	Ethyl Alc. 50%	Isop. Alc. 70%	Isop. Alc. 50%
Control Thermometer	+	+	+	+	+	+	+
20 sec.	+	+	+	+	+	+	+
40 sec.	+	+	+	+	+	+	+
60 sec.	+	+	+	+	+	+	+
80 sec.	+	0	+	+	+	+	+
100 sec.	+	0	+	+	+	+	+
120 sec.	0	0	+	+	+	+	+
3 min.	0	0	+	+	+	+	+
4 min.	0	0	+	+	+	0	+
5 min.	0	0	+	+	+	0	+
10 min.	0	0	+	0	0	0	0

+ = Growth after forty-eight hours.
0 = Growth after forty-eight hours.

TABLE VI.—RELATIVE EFFICIENCIES OF DISINFECTANT SOLUTIONS AGAINST S. AUREUS AFTER IMMERSION OF THERMOMETER SECTIONS COATED WITH A 50% CITRATED HUMAN PLASMA-S. AUREUS CULTURE MIXTURE

Immersion Time of Thermometer in Test Disinfectant, Min.	Iod. Soln. 2% I_2	Iod. Tr. 2% I_2	Ethyl Alc. 50%	Ethyl Alc. 70%	Isop. Alc. 70%	Isop. Alc. 50%
Control Thermometer	+	+	+	+	+	+
1	+	+	+	+	+	+
2	+	+	+	+	+	+
3	+	+	+	+	+	+
4	+	+	+	+	+	+
5	0	+	+	+	+	+
10	0	+	+	+	+	+

+ = Growth in forty-eight hours.
0 = Growth in forty-eight hours.

TABLE VII.—RELATIVE EFFICIENCIES OF DISINFECTANT SOLUTIONS AGAINST S. AUREUS AFTER IMMERSION OF THERMOMETER SECTIONS COATED WITH A 25% CITRATED HUMAN PLASMA-S. AUREUS CULTURE MIXTURE

Immersion Time of Thermometer in Test Disinfectant, Min.	Disinfectants Tested					
	Iod. Soln. 2% I_2	Iod. Tr. 2% I_2	Ethyl Alc. 50%	Ethyl Alc. 70%	Isop. Alc. 70%	Isop. Alc. 50%
Control Thermometer	+	+	+	+	+	+
1	+	+	+	+	+	+
2	+	+	+	+	+	+
3	0	+	+	+	+	+
4	0	+	+	+	+	+
5	0	+	+	+	+	+
10	0	+	+	+	+	+

+ = Growth in forty-eight hours.
0 = Growth in forty-eight hours.

media tubes containing a twenty-four hour growth of *S. aureus*.

Treated thermometer sections, not immersed in the disinfectant solution but placed immediately into the broth tube, were employed as positive controls.

CONCLUSIONS

1. In tests conducted against *S. hemolyticus* the iodine (tincture 2% and solution 2%) and alcohols, with the exception of ethyl alcohol 95%, killed the test organisms within twenty seconds.

2. Iodine tincture 2% (U. S. P. XIV) and iodine solution 2% (N. F. IX) killed *S. fecalis* within one hundred seconds, while the alcohol disinfectants did not kill within one hundred and twenty seconds.

3. The iodine preparations killed *E. coli* more quickly than did the alcohols, while ethyl alcohol 95% did not kill within one hundred and twenty seconds.

4. Iodine tincture (2%) killed *S. aureus* within eighty seconds, while iodine solution 2% required one hundred and twenty seconds to kill. Ethyl alcohol 95% was ineffective against *S. aureus* within ten minutes. The most efficient alcohol was isopropyl 70%, which killed *S. aureus* within four minutes, while the other alcohol disinfectants killed within ten minutes.

5. Tests conducted with a 50% plasma-*S. aureus* culture mixture revealed that iodine solution 2% killed *S. aureus* within five minutes. The other test disinfectants did not kill the test organism within ten minutes.

6. In similar tests using a 25% plasma-*S. aureus* culture mixture, iodine solution (2%) killed *S. aureus* within three minutes, while the other test disinfectants were ineffective within a ten-minute test period.

7. Iodine tincture U. S. P. or iodine solution N. F., widely used antiseptics which are readily available everywhere, were found to be more effective than either ethyl alcohol or isopropyl alcohol for the quick disinfection of clinical thermometers which were heavily infected with many of the commonly found bacteria.

WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

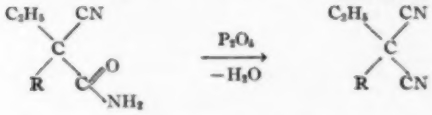
Triphenylmethyl mercaptan
Tetraphenylhydrazine
Tetraphenylarsonium chloride
Pulegol
2-Pentadecanone
Octyldiphenyl phosphate
Nitrosomethylurethane
2-Methyl-1,2-diamino propane
Guanosine deaminase
Guanase
Guanidine
2-Ethylanthraquinone
Adenase
Adenosine deaminase
2,4-Dihydroxypyrimidine
Butyraldehyde dibutyl acetal
Carboxy-*para*-benzoquinone
Ptelein
Protoanemonin
Camphorene

The Preparation of Some Dialkylmalononitriles*,†

By ROBERT F. DOERGE‡ and CHARLES O. WILSON§

A general procedure for the synthesis of dialkylmalononitriles is described and its application to the preparation of four substituted malononitriles is outlined for each compound.

IN THE course of synthetic work being done in the field of barbiturate compounds possibly having central nervous system depressant properties, it was necessary to have a convenient synthesis for dialkylmalononitriles. The alkylation of malononitrile itself results in a mixture of products and a poor yield of the substituted malononitrile (1). Furthermore, the preparation of malononitrile itself must be carefully controlled (2). In the present work the method of Errera and Berte (3) was used with certain modifications. By modifying their method in that the corresponding dialkylcyanoacetamide (4) was dehydrated to the dialkylmalononitrile, using phosphorus pentoxide as the dehydrating agent and working at reduced pressures and distilling the nitrile from the reaction mixture as it was formed, yields of over 80 per cent were consistently obtained.



An equal weight of clean dry sand was used to facilitate heat transfer, as was advocated by Dumas (5). The method of Errera and Berte (3) was to heat the dialkylcyanoacetamide with a little more than an equimolecular quantity of phosphorus pentoxide at 170–180°. They then allowed the reaction mixture to cool somewhat and poured off the nitrile that had been formed; then the remaining metaphosphoric acid was decomposed by adding water cautiously. Any remaining nitrile (which is insoluble in water) was then added to the previous yield.

In the present work the nitriles were identified by condensation with urea in a solution of sodium in dry ethyl alcohol. The imino derivative obtained was not isolated but was hydrolyzed directly to the corresponding barbituric acid derivative by refluxing with 20 per cent hydrochloric acid for one to two hours. This is a modification of the method used by Cope and Hancock (6).

Pertinent data for the compounds synthesized and the derivatives made are given in Table I.

EXPERIMENTAL

Diethylmalononitrile.—Diethylcyanoacetamide (10 Gm.) and phosphorus pentoxide (20 Gm.) were placed in a 250-cc. round-bottomed flask with an equal volume of clean dry sand and mixed by thorough shaking. The flask was then attached to a short (8 in.) air condenser which, in turn, was attached to a 250-cc. receiving flask which had an outlet to which a vacuum of 3–5 mm. was applied by using a Hyvac pump. The reaction flask was then heated by raising a bath of melted Wood's metal around it. The reaction period lasted about twenty to thirty minutes and was complete when no more nitrile distilled over. At the beginning of the reaction it was necessary to flash heat on the air condenser to prevent condensation of the nitrile at that point. The nitrile was then removed from the receiving flask by hot alcohol. The alcohol solution was then made cloudy with hot water and crystallization carried out in an ice-salt mixture. After one hour the crystals were filtered off and dried in a vacuum desiccator. The solid nitrile was obtained in a yield of 8 Gm. (93%) with a boiling point of 53°/5 mm. and a melting point of 42–43°. The literature (3) reports 44–45°.

Ethylisopropylmalononitrile.—Ethylisopropylcyanoacetamide (7.7 Gm.) and phosphorus pentoxide (14.2 Gm.) were placed in the reaction apparatus described for preparing diethylmalononitrile. The reaction was run in the same general manner. The nitrile was obtained as a high boiling water-white liquid in a yield of 5.8 Gm. (85.3%). The product boiled at 200–205°/760 mm. and 52–54°/4 mm.

Ethylisobutylmalononitrile.—Ethylisobutylcyanoacetamide (9.7 Gm.) and phosphorus pentoxide (29.1 Gm.) were caused to react in the same general manner described for the preparation of diethylmalononitrile. The product was obtained in a yield of 7.6 Gm. (87.7%) as a water-white liquid with a boiling point of 65–70°/5 mm.

Ethylisoamylmalononitrile.—Ethylisoamylcyanoacetamide (9.1 Gm.) and phosphorus pentoxide (18 Gm.) were reacted in the same general manner described for the preparation of diethylmalononitrile. The product was obtained in a yield of 6.8 Gm. (82.9%) as a water-white liquid with a boiling point of 88–92°/3 mm.

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TABLE I.—DERIVATIVES OF MALONONITRILE[a]

R	R′	B. P., ° C.	Yield, %	Molecular Formula	Derivative Prepared	M. P., ° C. Lit.	M. P., ° C. Found
C_3H_8	C_2H_5	42–43[b] (m. p.) 53/5 mm.	93	$C_7H_{10}N_2$	5,5-diethylbarbituric acid	191[c]	185–187
i-C_3H_7	C_2H_5	52–54/4 mm. 200–205/760 mm.	85	$C_8H_{12}N_2$	5,5-ethylisopropyl-barbituric acid	197–198[d]	195–196
i-C_4H_9	C_2H_5	65–70/5 mm.	88	$C_9H_{14}N_2$	5,5-ethylisobutyl-barbituric acid	174–176[e]	170–176
i-C_5H_{11}	C_2H_5	88–92/3 mm.	83	$C_{10}H_{16}N_2$	5,5-ethylisoamyl-barbituric acid	154–156[e]	153–154

[a] All melting and boiling points are uncorrected.
[b] Errera, G., and Berte, E., *Gaz. chim. ital.*, 26 II, 220(1896), report 44–45°.
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SUMMARY

1. A procedure for the synthesis of dialkylmalononitriles has been presented.

2. Four substituted malononitriles were prepared:

(*a*) Diethylmalononitrile.
(*b*) Ethylisopropylmalononitrile.
(*c*) Ethylisobutylmalononitrile.
(*d*) Ethylisoamylmalononitrile.

All except (*a*) have not been previously reported.

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Dry Basal Media for Rapid Microbiological Assay of Amino Acids*

By J. J. MAYERNIK and S. M. EWALD

A rapid, simplified method of preparing dry complete mixtures of basal media for the microbiological assay of amino acids is described. Also reported are modifications in the assay procedure using these media. From a practical operational standpoint, the assay is considerably more standardized and simplified than previous methods.

IN A CONTROL laboratory which is frequently required to perform many microbiological assays of a variety of amino acids, it is desirable that the assay procedures be as simplified, as highly standardized, and as rapid as possible. Stokes, *et al.* (1), contributed to the standardization and simplification of microbiological amino acid assays by developing one standard basal medium prepared from stock solutions for use with only two test organisms in the assay of 10 essential amino acid assays. Rockland and Dunn (2) suggested simplification in the method of covering assay tubes, in the use of an automatic pipette for the transfer of liquids, and in the use of a single dry mixture as a complete basal medium. Further elaboration on the preparation of a dry mixture in a ball mill was made by Camien and Dunn (3).

The present paper describes the further standardization and simplification of micro-

* Received April 25, 1951, from the Chemical Control Laboratories, Merck and Co., Inc., Rahway, N. J.

biological assays of amino acids by presenting: (*a*) a rapid, simplified method of preparing dry complete mixtures of basal media for the more common amino acids, and (*b*) modifications in the assay procedure of Stokes to facilitate the assay of routine samples.

PREPARATION OF DRY MIXTURES OF BASAL MEDIA

The composition of the basal medium for each amino acid is that of Stokes, *et al.* (1). Accurate quantities of the essential and accessory growth substances, omitting the amino acid to be assayed, are combined in the proportions shown in Table I, and blended in a Waring blendor for approximately five minutes. In this process the mixture is thoroughly blended, and also pulverized to a fine powder.

TABLE I.—COMPOSITION OF BASAL MEDIUM

Ingredient	Weight, Gm.	Ingredient	Weight, Gm.
DL-Alanine	2.0	DL-Threonine	2.0
L-Arginine	2.0	DL-Tryptophane	4.0
DL-Aspartic Acid	2.0	L-Tyrosine	2.0
L-Cystine	2.0	DL-Valine	2.0
DL-Glutamic Acid	2.0	Glucose	100.0
Glycine	2.0	Sodium acetate anhyd.	60.0
L-Histidine	2.0	KH_2PO_4	5.0
L-Hydroxyproline	2.0	K_2HPO_4	5.0
DL-Isoleucine	2.0	NaCl	0.1
DL-Leucine	2.0	$MgSO_4 \cdot 7H_2O$	2.0
DL-Lysine	2.0	$FeSO_4 \cdot 7H_2O$	0.1
DL-Methionine	2.0	$MnSO_4 \cdot 4H_2O$	0.1
DL-Norleucine	2.0	Adenine	0.1
DL-Phenylalanine	2.0	Guanine	0.1
L-Proline	2.0	Uracil	0.1
DL-Serine	2.0	Vitamin blend*	0.112

* For composition see Table II.

Salts, such as $MnSO_4 \cdot 4H_2O$, which consist of large crystals are first ground in a mortar before being combined with the other ingredients. To eliminate the necessity of individually weighing minute quantities of each of the required vitamins, a separate composite vitamin blend is prepared by combining the vitamins in a sodium acetate base in the proportions indicated in Table II and blending thoroughly in the Waring blendor for approximately five minutes. A single weight of 112 mg. ($^1/_{2,500}$ of the total weight) of this blend supplies the vitamins required for one amino acid mixture. The amount of sodium acetate contributed by the vitamin blend is negligible.

One complete mixture is sufficient to prepare 10 L. of final assay medium. The medium is soluble in cold water, except for a small quantity of cystine which dissolves almost completely on autoclaving. This slight remaining precipitate should be evenly distributed by shaking.

The dry mixtures, stored in tightly capped brown glass bottles at a temperature of 75° F. and a relative humidity of 30–60%, have been found to be stable on the basis of use for at least twelve months. As reported by Camien and Dunn (3) the solutions obtained from stored mixtures are slightly darker in color than those from freshly prepared amino acids.

PROCEDURE OF ASSAY

The assay procedure used was originally identical to that described by Stokes (1). However, to further simplify the work and for greater speed in performing routine assays of purified casein hydrolysates, the following changes were adopted.

Maintenance of Cultures and Preparation of Inocula.—Stab stock cultures of *S. faecalis* and *L. delburckii* are carried on Difco micro assay culture agar. For the preparation of inocula, transfers are made from stock cultures into Difco micro inoculum broth. After incubation, each culture is centrifuged, washed, and suspended in 100 cc. of sterile water.

Preparation of Standards and Samples.—The purity of each amino acid to be used as a standard should be established by an appropriate method such as phase solubility analysis (4). A stock solution of each is prepared in a concentration of 1,000 μg./cc. of the L-isomer. In order to eliminate the volume adjustment which is required for each tube when different quantities are pipetted from one standard solution, nine working solutions of increasing levels of the L-isomer are prepared from the stock solution. A standard curve is then established by pipetting, in duplicate, 1 cc. of each of

TABLE II.—COMPOSITION OF VITAMIN BLEND

Ingredient	Wt., Gm.
Thiamine hydrochloride	5.0
Pyridoxamine	10.0
Calcium pantothenate	5.0
Riboflavin	5.0
Nicotinic acid	5.0
p-Aminobenzoic acid	1.0
Biotin	0.005
Folic acid	0.1
Sodium acetate (anhyd.)	250.0
Total weight	281

the working solutions to a series of assay tubes. The refrigerated working solutions may be used for one week.

The solution of the sample is diluted to three appropriate levels and pipetted in the same manner as the standard working solutions. The use of 1-cc. serological "blowout" pipettes were found advantageous in increasing the speed of pipetting the standards and samples to the assay tubes.

Sterilization, Inoculation, and Incubation.—The assay tubes, containing only the solutions of the standards or samples, are covered with metal caps and autoclaved for ten minutes at 15 lb. pressure. The medium is prepared at single strength instead of double strength by dissolving 21.3 Gm. of the mixture per liter of water. It is sterilized in a Pyrex flask along with the assay tubes.

After cooling, the medium is inoculated with 1 cc. of the culture suspension per 100 cc. of medium, and shaken thoroughly. With a sterile automatic pipette, 4 cc. of the inoculated medium is delivered to each tube, bringing the total volume to 5 cc. The tubes are then incubated at 37° C. for sixteen to eighteen hours in a constant temperature water bath.

Determination of Microbial Response.—The response of the organisms to the amino acids is determined by measuring the turbidity with a Lumetron Photoelectric Colorimeter, Model 402E. A special drain tube adapter similar to the one designed by McMahan (5) is used for transmission measurements which are taken directly from the galvanometer scale.

DISCUSSION

By means of this method of preparation of dry basal media, many mixtures can be prepared easily and quickly with no need for expensive equipment. The mixtures do not have to be dried for blending, and one person can prepare all 10 media in less than an eight-hour working day.

Several pipetting operations have been combined in the procedure. The working standards and samples are pipetted in constant quantities so that volume adjustment with water is not required. Also, the medium is inoculated before being distributed to the test tubes in order to eliminate individual inoculation of each tube. This also tends to guarantee uniform inoculation of all tubes.

In determining microbial response, turbidimetric measurements, as an alternative to titrations, have made it possible to reduce considerably the incubation period of the assays. This is desirable in a control laboratory where the completion of large volumes of work in short periods of time is important. The use of a special drain tube adapter has eliminated the necessity of selecting photometrically matched test tubes for use in the photometer, and has also reduced the time required for transmission measurements.

TABLE III.—ASSAY RESULTS OBTAINED ON "AMINO ACIDS SOLUTION MERCK" BY USING MEDIA PREPARED FROM STOCK SOLUTIONS AND FROM DRY MIXTURES

Amino Acid	Media Prepared from Soln., Stokes Procedure, Gm./L.	Media Prepared from Dry Mixtures, Modified Procedure, Gm./L.
Arginine	2.2	2.2
Histidine	4.1	4.1
Isoleucine	7.2	6.9
Leucine	14.6	14.6
Lysine	9.9	9.6
Methionine	4.1	4.3
Phenylalanine	4.0	3.9
Threonine	2.0	1.9
Tryptophane	0.8	0.8
Valine	6.8	6.6

A constant temperature water bath is used, rather than a convection-type air incubator. Toennies and Gallant (6) have shown that the variations in bacterial growth occurring among identically charged tubes can be reduced to about one-third by the use of a water bath.

The reliability of the method using the media, organisms, and basic procedure of assay has been established by Stokes (1). Our modifications of this procedure have been primarily in the method of preparing media and in the techniques employed. Table III shows the agreement of assay results obtained by the Stokes procedure, using media prepared from stock solutions, with those obtained by the modified procedure, using media prepared from dry mixtures.

SUMMARY

The preparation of dry composite media for the determination of 10 amino acids has been described. The media are quickly and easily prepared and are stable for at least twelve months. Modifications in techniques in a procedure of assay employing these media are presented. These changes have been found to be time- and labor-saving and to be a considerable improvement over previous methods from a practical operational standpoint.

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A Phase Study of Castor Oil, Alcohol, and Water Miscibility*,†

By MURIEL R. LORAN‡ and EARL P. GUTH§

A phase diagram was drawn of the ternary system, castor oil, 95% alcohol, and water. The various ratios at which solutions of these three components can be prepared were determined from the diagram. This study was undertaken to illustrate the practical use of the phase rule in pharmacy in the preparation of solutions of two immiscible liquids, utilizing a third liquid which is miscible with both.

THE STUDY of a ternary system comprising alcohol, castor oil, and water was undertaken with the intent of investigating the use of the phase rule in practical pharmacy. Previous to this study, the ratios of each of the components necessary to produce a homogeneous system were determined by the pharmacist largely in an empirical manner. Although the empirical method has been successful, it is limited by its very nature. A phase study completely describes the system so that by simple inspection of a diagram all ratios at which the system is homogeneous can be quickly and accurately determined.

TABLE I.—TITRATION OF CASTOR OIL-WATER MIXTURES WITH ALCOHOL

Castor Oil, Cc.	Water, Cc.	Alcohol, Cc. Required
5 cc.	0.2	4.1
	0.3	8.3
	0.5	14.1
	1.0	24.1
	2.0	42.4
	3.0	70.0
1 cc.	1.0	9.9
	2.0	23.8
	3.0	40.51
	4.0	57.61
	6.0	83.71
	10.0	119.81
	18.0	178.01
	34.0	278.11

The phase rule of Willard Gibbs (1) for this kind of system is $F = C - P$ where F equals the degrees of freedom, P the number of phases in equilibrium, and C the number of components comprising the system. The degrees of freedom are defined as the number of variable factors—temperature, pressure, and concentration of the components—which must be arbitrarily fixed in order that the condition of the system may be perfectly defined. The greater the degrees of freedom, the larger the variability, and it is the merit of the phase rule that the state of a system is defined entirely by the relation existing between the number of the components and the phases present, no account being taken of the molecular complexity of the substances involved nor any assumption made as to the constitution of matter.

METHOD

The components of the system investigated are U. S. P. castor oil, 95% alcohol, and distilled water. Five cubic centimeters of castor oil was added to a 250-cc. flask and to this was added 0.2 cc. of distilled water and the mixture titrated with alcohol until a clear solution was obtained. The procedure was repeated with 0.3, 0.5, 1.0, 2.0, and 3.0 cc. of water (Table I).

TABLE II.—CALCULATED CONCENTRATION IN CC. OF WATER AND CASTOR OIL IN EQUILIBRIUM WITH 100 CC. OF 95% ALCOHOL

Water, Cc.	Castor Oil, Cc.
4.88	122.0
6.03	60.2
7.1	35.4
8.66	20.7
9.44	11.8
10.0	7.15
10.1	10.1
13.2	4.2
14.8	2.46
17.4	1.73
19.1	1.19
20.6	0.834
24.7	0.562
28.0	0.360

One cubic centimeter of castor oil was then added to a 500-cc. flask and to this was added 1 cc. of distilled water and the mixture titrated with alcohol until a clear solution was obtained and the procedure was repeated with 2, 3, 4, 6, 10, 18, and 34 cc. of water (Table I).

Because of the difficulty of observing the end point by macroscopic observation, the Fisher Electrophotometer was used. Transmission of 98% was taken as the end point using a "B" filter.

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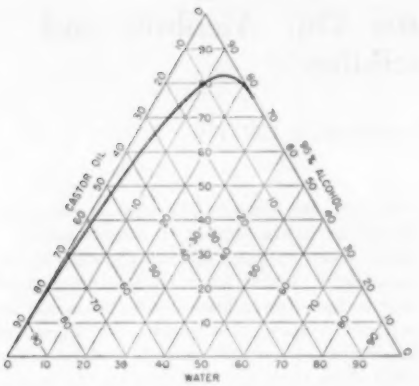

Figure 1

CALCULATIONS

The number of cubic centimeters of water and oil, respectively, at equilibrium with 100 cc. of alcohol was calculated and then the number of cubic centimeters multiplied by the specific gravity to obtain the weight of each component in grams. The weight fraction was then calculated and multiplied by 100 to obtain the percentage of each component by weight (Table II). The points were then plotted on a triangular graph (Fig. 1) of which each side represents 0 to 100% so that at any point on the graph the percentages of each of the components in equilibrium can be determined (Table III). Practical values derived from the phase diagram of each component, which when mixed will give a homogeneous solution, are shown in Table IV.

RESULTS

At any point inside the curve, the system shall exist as two phases in equilibrium with each other and at any point above the curve the system is homogeneous and consists of only one phase.

CONCLUSIONS

1. The system castor oil, alcohol, and water was investigated by means of the phase rule.

2. A phase diagram of the system was produced.

3. At all ratios above the curve the system is homogeneous and at all ratios below the curve the system exists as two phases in equilibrium.

TABLE III.—PER CENT BY WEIGHT OF EACH COMPONENT TO PRODUCE A HOMOGENEOUS SYSTEM

Water	Alcohol	Castor Oil
2.45	41.3	56.2
4.50	56.3	39.2
5.78	66.7	37.5
7.86	74.3	17.8
9.20	79.8	11.0
9.93	80.6	9.47
10.14	83.1	6.76
13.3	82.6	4.10
14.9	82.5	2.60
17.2	81.2	1.60
18.7	80.2	1.10
19.9	79.5	0.60
23.0	76.5	0.50
25.4	74.3	0.30

TABLE IV.—PRACTICAL VALUES DERIVED FROM THE PHASE DIAGRAM. PERCENTAGE (W/W) OF EACH COMPONENT WHICH WHEN MIXED WILL GIVE A HOMOGENEOUS SOLUTION

Castor Oil	Water	95% Alcohol
5.0	10.0	85.0
10.0	8.0	82.0
15.0	8.0	77.0
20.0	6.0	74.0
25.0	5.0	70.0
35.0	4.0	61.0
40.0	4.0	56.0
45.0	3.0	52.0
50.0	3.0	47.0
75.0	1.0	24.0
25.0	5.0	70.0
5.0	10.0	85.0
1.0	15.0	84.0
4.0	20.0	79.0
0.2	25.0	74.8
94.0	1.0	5.0
89.0	1.0	10.0
84.0	1.0	15.0
79.0	1.0	20.0
73.0	2.0	25.0
68.0	2.0	30.0
63.0	2.0	35.0
57.0	3.0	40.0
52.0	3.0	45.0
47.0	3.0	50.0
1.0	14.0	85.0

4. There are varying ratios at which a homogeneous sysem can be prepared and these points can be determined from the diagram.

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7-Methyl-Thiocinchoninamide*

By ALICE G. RENFREW

The preparation of 7-methyl-thiocinchoninamide is described. Evidence is given for the stability of this compound in inert solvents; however, decomposition occurred at 85° in the presence of dialkylaminoalkylamines.

THE 4-thioamide of 7-methylquinoline was prepared in relation to the testing program sponsored by the Chemotherapy Center of Tropical Medicine. It was hoped that 7-methyl-thiocinchoninamide would serve as an intermediate in the synthesis of substituted amidines; however, under the conditions employed, this thioamide was converted back to 4-cyano-7-methylquinoline when heated with alkyl amines of high molecular weight. Schlatter (1) has commented on similar reactions of certain thioamides.

The preparation of quinoline derivatives from 2- and 4-cyanoquinolines has been described by Coates, *et al.* (2). In the present work the procedure of Olin and Johnson (3) provided a very simple synthetic method.

7-METHYL-THIOCINCHONINAMIDE (CC1029)[1]

Coates, *et al.* (2), described the formation of thiocinchoninamide by the addition of hydrogen sulfide to the cyano group of 4-cyanoquinoline. In preparing the corresponding derivative of 7-methylquinoline, an adaptation of the method of Olin and Johnson (3) was used. A chilled solution of 10 cc. of triethanolamine in 75 cc. of methanol was saturated with hydrogen sulfide. After addition of a solution of 18 Gm. of 4-cyano-7-methylquinoline (4) in 150 cc. of dry benzene, the stream of hydrogen sulfide was continued for an hour. After a further eighteen hours at room temperature, 11 Gm. of a crystalline product was collected on a filter. The mother liquor plus 3 cc. of triethanolamine was again saturated with hydrogen sulfide; after thirty-six hours a second crop of 6.7 Gm. was collected. The crude yield was 80% of the theoretical.

7-Methyl-thiocinchoninamide was crystallized from 95% ethanol; about 70 cc. of solvent per gram of crude thioamide was used for extraction and the alcoholic mother liquor was recirculated. After several subsequent recrystallizations, the product melted at 209° with decomposition.

Anal.—Calcd. for $C_{11}H_{10}N_2S$: N = 13.86; S = 15.84. Found: N = 13.67; S = 15.76.

Reaction of 7-Methyl-thiocinchoninamide with High Molecular Weight Amines.—This thioamide had been prepared as a possible intermediate in the synthesis of quinoline amidines. Although the thioamide was stable when heated in a liquid medium at fairly high temperatures, it decomposed when heated with several typical organic amines.

(*a*) After refluxing a suspension in toluene for one hour it was possible to recover 7-methyl-thiocinchoninamide unchanged.

(*b*) However, when the thioamide was heated at 85° in diethylaminoethylamine, a strong evolution of hydrogen sulfide occurred and 4-cyano-7-methylquinoline was recovered.

(*c*) With novol diamine[2] no reaction occurred on very mild heating; decomposition to 4-cyano-7-methylquinoline occurred with more vigorous heating. The 4-cyano-7-methylquinoline was identified by mixed melting point determination and by solubility properties.

SUMMARY

7-Methyl-thiocinchoninamide was prepared from 4-cyano-7-methylquinoline by the addition of hydrogen sulfide in the presence of triethanol amine. Simple attempts to convert this to an amidine were not successful.

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* Received May 31, 1951, from the Department of Research in Organic Chemistry, Mellon Institute, Pittsburgh, Pa.

[1] The number assigned the compound by the testing program of the Chemotherapy Center for Tropical Diseases under the National Research Council.

[2] 1-Methyl-4-diethylaminobutylamine.

The Preparation of Some 4,6-Dithiobarbituric Acids*,†

By ROBERT F. DOERGE‡ and CHARLES O. WILSON§

Preparative methods for four 5,5-disubstituted-4,6-diiminobarbituric acids and for four 5,5-disubstituted-4,6-dithiobarbituric acids are described. Seven of the compounds reported are new. One of the compounds, 5,5-diethyl-4,6-dithiobarbituric acid, was tested pharmacologically and found to be unsuitable for possible use as a general anesthetic because of its inordinately long duration of action and the production of tremors.

UNTIL 1935 thiobarbituric acids were used only as intermediates in the preparation of the corresponding oxygen analogs. In 1935 Miller, *et al.* (1), and Tabern and Volwiler (2) reported the synthesis of various 2-thiobarbituric acids and their use as hypnotics of short duration of action. Of the group, the ethyl-(1-methylbutyl) derivative as the sodium salt has found use in medical practice under the name "Pentothal sodium."

Henze and Smith in the United States (3) and Carrington in England (4), using phosphorus trisulfide or phosphorus pentasulfide, were able to replace the oxygen of the barbituric acids by sulfur. Henze reported the preparation of 5,5-diethyl-2,4,6-trithiobarbituric acid and 5-phenyl-5-ethyl-2,4,6-trithiobarbituric acid. He also reported that these compounds possessed no hypnotic properties. Carrington prepared the 2-thio, 2,4,-dithio, and 2,4,6-trithio derivatives of a series of barbituric acids that are well-known hypnotics. However, no report of the pharmacological action of the thio-derivatives has been found in the literature surveyed.

Not until the report by Wilson and Boothe in 1945 (5) of 4-thiobarbituric acids were compounds with sulfur in other positions than the 2-position reported in the literature. These compounds were prepared by treatment of the corresponding 4-imino compound with hydrogen sulfide under pressure and heat in a sealed bomb (6). The compounds reported by them had a depressant action (7).

This work has been extended to the synthesis of 4,6-dithiobarbituric acids by treating the corresponding 4,6-diimino compound with hydrogen sulfide under heat and pressure in a sealed bomb using a modification of the method reported by Boothe and Wilson. The 4,6-diimino compounds were prepared by the condensation of the proper disubstituted malononitrile (8) with urea in the presence of sodium ethoxide (see Eq. 1).

All of the diimino compounds melted with decomposition and were difficult to crystallize to a constant melting point. All of the diimino compounds were identified by hydrolysis to their oxygen analog by refluxing a sample with 10% hydrochloric acid for four hours (see Eq. 1).

The 4,6-dithiobarbituric acids were oxidized to the corresponding oxygen analog to establish their identity, using Conrad's method (9).

EXPERIMENTAL[1]

5,5-Diethyl-4,6-diiminobarbituric Acid.—Sodium (2.3 Gm.) was dissolved in anhydrous alcohol (25 cc.) and added to diethylmalononitrile (8) (6.1 Gm.) and urea (4.5 Gm.) in a 100-cc. pressure flask; a clear solution resulted in a short time. The reaction flask was sealed and heated with its contents in a boiling water bath for four hours. The container for the boiling water was an iron pipe about 6 in. in diameter and 18 in. long with an iron plate welded across one end to make it watertight. This precaution was taken so that in case of an explosion the force would be directed up. Also, the entire apparatus was loosely covered in order to minimize the loss of water by evaporation. After the heating period the flask was allowed to stand unopened at room temperature overnight. The flask was then opened and the contents transferred to a distillation flask. The mixture was then taken to dryness by vacuum distillation by a water pump and a minimum of heat from a steam bath. The residue was washed with a little ether to remove any unreacted diethylmalononitrile. The remaining solid was stirred thoroughly with 10 cc. of water and filtered to remove the sodium acetate and any unreacted urea. The compound was filtered off and washed on the filter paper with another 10-cc. portion of water. The 5,5-diethyl-4,6-diiminobarbituric acid was dried in a vacuum desiccator. A yield of 8 Gm. (87.9%) was obtained and the product melted at 267–269° with decomposition. The literature (10) reports 272° with decomposition.

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† Abstracted from a thesis presented to the Graduate Faculty of the University of Minnesota by Robert F. Doerge in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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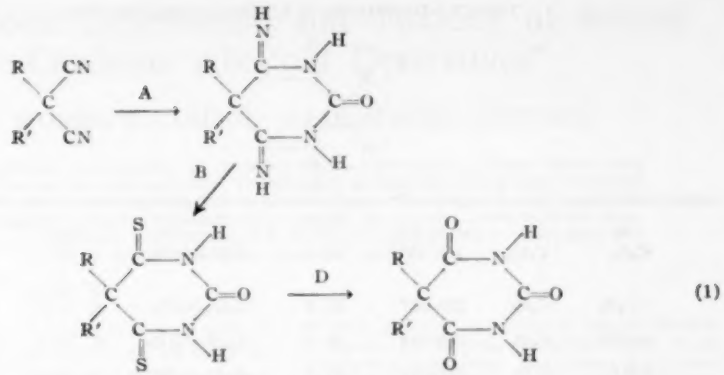
[1] All melting points and boiling points are uncorrected,



(1)

Equation 1.—Summary of the reaction series.

R, ethyl. R′, ethyl, isopropyl, isobutyl, or isoamyl. A, urea and sodium ethoxide. B, H_2S under pressure and heat. C, reflux with 10% hydrochloric acid. D, oxidation with nitric acid.

5,5-Ethylisopropyl-4,6-diiminobarbituric Acid.—Sodium (2.3 Gm.), anhydrous alcohol (25 cc.), ethylisopropylmalononitrile (6.8 Gm.), and urea (4.5 Gm.) were used in the same procedure employed for preparing 5,5-diethyl-4,6-diiminobarbituric acid. The yield was 5 Gm. (51%), having a melting point of 265–268° with decomposition.

5,5-Ethylisobutyl-4,6-diiminobarbituric Acid.—Ethylisobutylmalononitrile (7.5 Gm.) was used in a reaction mixture similar to that employed for the two preceding 4,6-diiminobarbituric acids. The yield was 5.5 Gm. (52.3%) and had a melting point of 270–273° with concomitant decomposition.

5,5-Ethylisoamyl-4,6-diiminobarbituric Acid.—Ethylisoamylmalononitrile (8.2 Gm.) was used in a reaction mixture similar to that previously described. The yield was 6.2 Gm. (55.4%), which melted at 260–263° with decomposition.

5,5-Diethyl-4,6-dithiobarbituric Acid.—Absolute alcohol (150 cc.) and 5,5-diethyl-4,6-diiminobarbituric acid (3 Gm.) were charged to a stainless steel liner of a pressure bomb.[1] The liner was capped and placed in the bomb and evacuated for three to five minutes by using a water-pump aspirator. The slight vacuum was not broken until hydrogen sulfide under pressure was led into the vessel. A pressure of about 50 lb./sq. in. was built up by rocking the apparatus. The valves were then tightly closed and heat was applied by means of the electrical unit. A temperature of 125–135° was maintained for eleven hours. At this temperature the pressure was about 600 lb./sq. in. The apparatus was then allowed to cool overnight. The remaining pressure was then cautiously released to the outside atmosphere and the water-pump aspirator was again attached to the inner bomb liner and the contents evacuated of gas for three to five minutes. The pressure was then allowed to equalize and the contents were removed from the liner. The liner was thoroughly rinsed with alcohol and the rinsings added to the yellowish reaction mixture. This solution was then filtered and evaporated to dryness. The residue was then dissolved in 200 cc. of 2% sodium hydroxide solution with the aid of slight heat if necessary. This solution was filtered and acidified to *p*H 8 (Universal Indicator Paper) by bubbling carbon dioxide through it. The fine yellow crystals were filtered off and recrystallized by dissolving in cold alcohol. This solution was then warmed and hot water was added, drop by drop, until cloudiness resulted. This material was then placed in the refrigerator overnight to allow crystallization. The yield was 1.5 Gm. (41.7%) and the melting point was 163–164°.

Anal.—Calcd. for $C_8H_{12}ON_2S_2$: S, 29.4; N, 12.95; Found: S, 29.5, 29.0; N, 12.64.

5,5-Ethylisopropyl-4,6-dithiobarbituric Acid.—5,5-Ethylisopropyl-4,6-diiminobarbituric acid (3 Gm.) and absolute alcohol (150 cc.) were placed in the reaction apparatus described for preparing 5,5-diethyl-4,6-dithiobarbituric acid. The reaction and manipulation were carried out in the same general manner previously described. A yield of 1.1 Gm. (31.2%) was obtained which had a melting point of 226–227°.

Anal.—Calcd. for $C_9H_{14}ON_2S_2$: S, 27.8; Found: S, 27.5, 27.5.

5,5-Ethylisobutyl-4,6-dithiobarbituric Acid.—5,5-Ethylisobutyl-4,6-diiminobarbituric acid (3 Gm.) in absolute alcohol (150 cc.) was converted to the corresponding 4,6-dithiobarbituric acid by the previously described method. A yield of 1.1 Gm. (36.7%) was obtained. The recrystallized compound had a melting point of 213–214°.

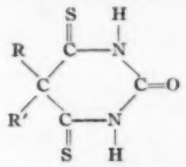
Anal.—Calcd. for $C_{10}H_{16}ON_2S_2$: S, 26.3; Found: S, 26.0, 26.0.

5,5-Ethylisoamyl-4,6-dithiobarbituric Acid.—5,5-Ethylisoamyl-4,6-diiminobarbituric acid (3 Gm.) in absolute alcohol (150 cc.) was converted to the corresponding 4,6-dithiobarbituric acid by the previously described method. A yellow, crystalline product was obtained in a yield of 1.6 Gm. (53.3%). The melting point was 240–241°.

Anal.—Calcd. for $C_{11}H_{18}ON_2S_2$: S, 24.8; Found: S, 24.5, 24.5.

[1] Specially constructed by American Instrument Company, Silver Spring, Md.

TABLE I.—DERIVATIVES OF 4,6-DITHIOBARBITURIC ACID



R	R'	M. P., °C.	Yield, %	Molecular Formula	Analyses, % Calcd.	Found
C_2H_5	C_2H_5	163–164	41.7	$C_8H_{12}N_2OS_2$	S, 29.4	29.5
						29.0
					N, 12.95	12.64
i-C_3H_7	C_2H_5	226–227	31.2	$C_9H_{14}N_2OS_2$	S, 27.8	27.5
						27.5
i-C_4H_9	C_2H_5	213–214	36.7	$C_{10}H_{16}N_2OS_2$	S, 26.3	26.0
						26.0
i-C_5H_{11}	C_2H_5	240–241	53.3	$C_{11}H_{18}N_2OS_2$	S, 24.8	24.5
						24.5

5,5-Diethyl-4,6-dithiobarbituric acid was tested for pharmacological activity by Dr. K. K. Chen (11). The results reported are not to be considered as conclusive because only one compound has been tested and reported on. It may be that other compounds will have a shorter activity, but it appears that compounds having sulfur in the 4- or 6-positions are characterized by tremors or convulsions. In a dose of 30 mg./Kg., the compound caused a general anesthesia accompanied by tremors for an average of three hundred and sixty minutes. The median anesthetic dose was found to be 27.7 ± 3.9 mg./Kg. and the median lethal dose was shown to be 77.0 ± 5.0 mg./Kg. The compound was considered to be of no practical value because of the length of action, and because of the accompanying tremors.

SUMMARY

1. Four 5,5-disubstituted-4,6-diiminobarbituric acids were prepared, three of which are new compounds.

2. Four 5,5-disubstituted-4,6-dithiobarbituric acids were prepared, all of which are new compounds.

3. 5,5-Diethyl-4,6-dithiobarbituric acid was tested for pharmacological activity and was considered to be of no practical value as a general anesthetic because of the length of action and because of the accompanying tremors.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

Saccharic acid
Sodium tauroglycocholate
Stachyose
D-Talose
α-Thujene
Leucylglycylglycylglycine
D-Mannonic acid
α-Methyl maltoside
2,6-Dihydroxyquinoline
3,5-Diiodothyronine
Gentiobiose
L-Gulose
Camphorene
5-Chlorosalicylaldehyde
Cholesteryl linoleate
Copper nucleonate
Tincture of Boletus
Argininic acid
Piperidone
Opianic acid

The Blood Distribution and Toxicity of Several Cinchona Alkaloid Derivatives*

By WARNER W. CARLSON† and LEONARD H. CRETCHER

The distribution of a number of cinchona alkaloid derivatives between blood cells and plasma has been determined. The presence of hydroxyalkyl groups increased the proportion of the drug found in the plasma phase. Binding of the various derivatives by blood cells *in vitro* was found to parallel the systemic toxicity of the compounds. The chronic oral toxicity of hydroxyethylapocupreine to white mice was determined by the drug-diet method.

NUMEROUS investigations have been made of the distribution of cinchona alkaloids, especially quinine, between blood cells and plasma. The distribution of the alkaloid apparently is subject to factors introduced by variations in experimental technique, for Goodman and Gilman (1) report cell-to-plasma ratios varying from 1:3 to 1:0.2. Rona and Bloch (2), and more recently Ceithaml and Evans (3), have investigated various of these factors, among which *p*H was found to be especially important. As pointed out by Fulton (4), however, in general it is agreed that *in vitro* the cell concentration of quinine, and most cinchona derivatives, is higher than that in the plasma. In the present investigation, distribution in whole beef blood *in vitro* has been determined for quinine, hydroxyethylapocupreine, and several other cinchona derivatives.

Hogan and Eagle (5) studied a series of phenylarsenoxides as to binding of the drugs by red blood cells (rabbit) *in vitro* in comparison with the values obtained for relative systemic toxicities (white mice) of the compounds. It was found that binding of the arsenicals by red blood cells *in vitro* was a model for their combination with tissues *in vivo*, and furthermore that the degree to which the drugs were taken up by blood cells *in vitro* so closely paralleled their systemic toxicity as to provide a reliable measure of that factor. In a similar way the degree of binding of cinchona derivatives by blood cells *in vitro* has now been compared with the systemic toxicities of these compounds. Additional data also are given on the toxicity of hydroxyethylapocupreine.

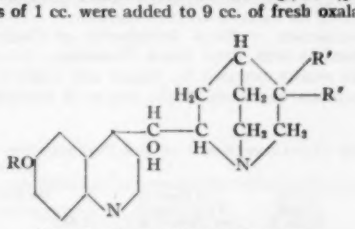
EXPERIMENTAL

Methods and Materials.—The cinchona alkaloid derivatives employed were selected from the group of drugs synthesized by this Department during the course of a cooperative search, with Maclachlan, Bracken, and their co-workers at Mercy Hospital, Pittsburgh, for cinchona derivatives useful in the treatment of pneumonia. For reviews of this work see Renfrew and Cretcher (6), and Maclachlan, Johnston, Bracken, and Pierce (7).

In the studies on blood distribution of the drugs, extraction of the alkaloids from blood cells or plasma, and their estimation nephelometrically as silicotungstates, was carried out by the method of Kyker, Webb, and Andrews (8). Extracted alkaloid was redissolved in that volume of acid which trial experiments indicated would yield silicotungstate suspensions in the turbidity range most favorable for measurement in the photoelectric nephelometer, a concentration correction being applied to the final result.

The chronic toxicity to white mice of quinine and hydroxyethylapocupreine was determined by the drug-diet method, essentially as described by Bratton (9). The two drugs (as free base) were prepared in powdered Purina dog chow. The drug-diets were placed in the inner member of a double food cup, thus allowing spilled food to be recovered. After being air-dried and sifted, recovered diet was weighed and allowance accordingly made in estimating daily food intake.

Distribution of Cinchona Derivatives in Whole Blood in Vitro.—Solutions of the various derivatives (I) as free bases were prepared in 10% aqueous alcohol in a concentration of 200 mg./L. Quantities of 1 cc. were added to 9 cc. of fresh oxalated beef blood contained in centrifuge tubes, giving an alcohol concentration of 1%. The final mixtures, with a drug concentration (free base) of 20 mg./L. were kept at room temperature for one-half hour, during which time they were inverted frequently. Trial experiments indicated that equilibrium was reached within approximately fifteen minutes. The mixtures were then centrifuged and the drug content of cells and plasma determined. The results, summarized in Table I, are similar to those of Fulton



(I). Cinchona Alkaloid Structure

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TABLE I.—DISTRIBUTION OF CINCHONA DERIVATIVES IN WHOLE BEEF BLOOD

	Drug	Structure R	R'	R''	Distribution Cells, %	Plasma, %
1	Optochin	CH_3CH_2	—H	$—CH_2CH_3$	86	14
2	Hydroquinine	CH_3—	—H	$—CH_2CH_3$	80	20
3	Ethylapocupreine	CH_3CH_2—	=CHCH$_3$	=CHCH$_3$	78	22
4	β-Chloroethylapocupreine	$ClCH_2CH_2$—	=CHCH$_3$	=CHCH$_3$	77	23
5	Quinine	CH_3—	—H	$—CH=CH_2$	70	30
6	β-hydroxyethylapocupreine	$HOCH_2CH_2$—	=CHCH$_3$	=CHCH$_3$	57	43
7	β-glycerylapocupreine	$(HOCH_2)_2CH$—	=CHCH$_3$	=CHCH$_3$	48	52

(4) and Shaw (10) in that they again demonstrate a relationship between structure and the degree to which a drug is taken up by blood cells. In particular, as might be expected, those compounds containing the solubilizing hydroxyalkyl groups were found to have an increased preference for the plasma phase.

Rona and Bloch (2) showed that the adsorption of cinchona alkaloids by blood cells is reversible. The decreased tendency towards adsorption shown by the hydroxyalkylated derivatives was accompanied by an increased ease with which the compounds could be washed away from the blood cells, as indicated in the following experiment. Beef blood samples containing quinine and hydroxyethylapocupreine in a concentration of 20 mg./L. were prepared as described previously. The mixtures were allowed to stand for fifteen minutes with frequent inversion, after which they were centrifuged, the plasma layers removed for analysis, and the blood cells resuspended in a volume of physiologic saline equal to that of the plasma removed. The mixtures were inverted frequently during fifteen minutes, centrifuged, and the cell and saline layers removed for analysis. It was found that the saline wash removed 52% of the alkaloid in the case of quinine, and 68% when hydroxyethylapocupreine was employed as the test substance. Because of the initial differences in cell-plasma distribution, this resulted in the ultimate retention by the cells of 33% of the originally bound alkaloid in the case of quinine, but only 18% for the hydroxyethyl derivative.

Comparison of Blood Distribution of Cinchona Derivatives with Their Acute Toxicities.—In view of the results obtained by Hogan and Eagle (5) a comparison was made of the degree of binding of cinchona derivatives, by bovine blood cells *in vitro*, with the systemic toxicity of these compounds in white mice. The results, which are given in Table II, suggest that for cinchona derivatives, as for the arsenicals, the degree of binding of the drugs by blood cells *in vitro* may possibly serve as a model for binding by body tissues *in vivo*, and hence as a measure of the systemic toxicity of the compounds. Since cinchona alkaloids undergo extensive metabolic degradation, it is recognized that some doubt can exist as to how completely the observed toxicities were due to the actions of intact drugs. However, it should be pointed out that the toxicity values given are correctly described as acute, for mice which were to die did so within a maximum period of four hours. In connection with the above results, the degree of binding of the drugs by blood cells was not found to be related to the hemolytic activity of the compounds. Hemolysis by these derivatives *in vitro* was not observed to become appreciable until concentrations at least tenfold greater (ca. 200 mg./L.) were reached, and at those levels no correlation between degree of binding and red cell destruction could be observed. In addition, study of the data of Renfrew and Cretcher (6) on the bacteriostatic potencies of these derivatives revealed an absence of relationship to relative degree of binding of the compounds by blood cells.

Comparative Toxicities of Quinine and Hydroxyethylapocupreine.—From Table II it is seen that the LD_{50} values for quinine and hydroxyethylapocupreine, following intraperitoneal injection of their dihydrochloride salts into white mice, are, respectively, 0.245 Gm. and 0.460 Gm./Kg. body weight. Values for acute oral toxicity in mice are higher; for hydroxyethylapocupreine, Bracken, *et al.* (11),

TABLE II.—COMPARISON OF THE DISTRIBUTION OF CINCHONA DERIVATIVES IN VITRO WITH THEIR ACUTE SYSTEMIC TOXICITY

Drug (From Table I)	Per Cent of Drug Bound by Blood Cells	Per Cent of White Mice Killed by Intraperitoneal Injection of Drug in Indicated Amounts[a] 4 Mg.	5 Mg.	6 Mg.	8 Mg.	9 Mg.	10 Mg.
1	86	37(*a*)	89(*b*)	100	...	...	...
2	80	...[b]	...	...	...	...	...
3	78	16	72(*e*)	88(*f*)	...	...	...
4	77	17(*c*)	70(*d*)	100	...	...	...
5	70	7	52(*g*)	87(*h*)	...	...	...
6	57	...	...	...	10	43(*i*)	83(*j*)
7	48	...	...	...	...	13(*k*)	70(*l*)

[a] The amounts indicated are mg. of drug per 20-Gm. mouse. These results were obtained by Maclachlan, Bracken, and their co-workers at Mercy Hospital, Pittsburgh (11, 13, 14). Representative results, showing the number of mice killed out of the total number used in each experiment, are: (*a*) 22/60; (*b*) 40/45; (*c*) 5/30; (*d*) 21/30; (*e*) 94/130; (*f*) 75/85; (*g*) 109/210; (*h*) 85/95; (*i*) 13/30; (*j*) 25/30; (*k*) 4/30; (*l*) 21/30.

[b] No. 2 (hydroquinine) was found to have low pneumococcicidal powers and to be highly toxic to mice, and hence was not further studied. Specific toxicity figures were not determined.

TABLE III.—CHRONIC ORAL TOXICITY OF QUININE AND HYDROXYETHYLAPOCUPREINE TO WHITE MICE

No. Mice	Drug in Diet, %	Diet Intake, Gm./Kg./Day	Drug Intake, Gm./Kg./Day	Dead/Total in 20 Days	Average Day of Death	Weight Change, %
			Quinine			
5	0.25	178	0.45	0/5	..	+13
5	0.50	168	0.84	0/5	..	+14
5	1.0	147	1.47[a]	1/5	11	−7
5	2.0	131	2.62	2/5	8	−29
			Hydroxyethylapocupreine			
5	0.25	190	0.48	0/5	..	+33
5	0.50	181	0.90	0/5	..	+35
5	1.0	170	1.70	0/5	..	+15
5	2.0	158	3.16[a]	0/5	..	−2
			Controls			
5	...	197	...	0/5	..	+36

[a] Maximum tolerated intake.

give 2.2 Gm./Kg. as the LD_{50} value for the dihydrochloride salt, and 3.0 Gm./Kg. for the free base administered as a suspension in gum acacia solution. Seeler, Dusenberry, and Malanga (12) determined the acute oral toxicity of quinine sulfate in mice to be approximately LD_{50} 1.0 Gm./Kg. body weight. To complete these data we have compared the chronic oral toxicites of these drugs to white mice by the drug-diet method. Male white mice in the weight range 14–19 Gm. were employed. The study was continued for twenty days, the mice being weighed at frequent intervals. The results, recorded in Table III, demonstrate that the chronic oral toxicity of hydroxyethylapocupreine to white mice is somewhat less than half that of quinine, this ratio being essentially the same as that noted previously for values for acute intraperitoneal and acute oral toxicity.

SUMMARY

The distribution of a number of cinchona alkaloid derivatives in whole blood has been determined, and the relative degree of binding of the compounds by blood cells has been related to their acute toxicities to white mice.

By the drug-diet method, the chronic toxicity to white mice of hydroxyethylapocupreine was found to be slightly less than one-half that of quinine (methyl cupreine).

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Notes

A Note on the Isolation of *l*-Inositol from *Euphorbia pilulifera*, L.*†

By FLOYD P. HALLETT‡ and LLOYD M. PARKS

ALTHOUGH *meso*-inositol has been encountered as a constituent of many plants, its optically active isomers rarely have been found except as their monomethyl ethers. The occurrence of small amounts of *l*-inositol in *Sonchus arvensis*, L. (sow thistle) was reported in 1925 (1). This report of its isolation from *Euphorbia pilulifera*, L. marks the second time that *l*-inositol has been encountered free in nature. In the course of work on isolation of the antispasmodic principle of this plant (2), minute amounts of *l*-inositol were obtained on prolonged standing (six months) of methyl and ethyl alcohol extracts of the crude drug, from which extraneous material had been removed by precipitation with lead acetate, ether, or Na_2SO_4. Subsequently it was found that larger amounts of the crystalline compound could be obtained by hot water extraction of the drug. The aqueous extract, when treated as described, yielded *l*-inositol in an amount equivalent to 0.5% by weight of the crude drug.

EXPERIMENTAL

Finely ground *E. pilulifera* (125 Gm.) was macerated with 1 L. of boiling water for one-half hour. The mixture was strained and the marc washed thoroughly with warm water. The combined aqueous extract and washings were reduced to 600 cc. using a flash evaporator. The concentrated solution was treated under an atmosphere of nitrogen with a hot saturated solution of lead acetate (15 cc.). After one hour the precipitate was removed by centrifuging. The clear yellow solution was passed through a column of Amberlite IR-120 resin (H form) to remove excess lead and other cations. The effluent was concentrated *in vacuo* to about 25 cc., and 150 cc. of absolute alcohol was added to precipitate considerable gummy material. The alcoholic solution was decanted and allowed to stand two days. It was again decanted from a slight additional amount of gummy material and then seeded with crystals obtained from the clarified alcoholic extracts (above). Slow crystallization of *l*-inositol began within several hours and was completed in two days. The crystalline material was removed by filtration, washed with ethanol, treated with Norit in boiling 92% methanol, and recovered from the filtered solution by evaporation. It was then recrystallized from absolute methanol.

The dried crystalline material sintered at 210° and melted with decomposition between 245 and 250°. It was slightly soluble in hot methanol and very slightly soluble in hot ethanol. From methanol the crystals were tetrahedral prisms; from ethanol they were octahedral. A Scherer test for inositols (3) was positive. The optical rotation was *levo*; $[\alpha]_D^{25} = -64.1°$ (water). Tanret (4) −65°, Heilbron (5) −64.1°.

Anal.—Calcd. for $C_6H_{12}O_6$: C, 40.00; H, 6.70. Found: C, 39.59; H, 6.66.

Acetylation yielded a small amount of amorphous derivative which could not be obtained in crystalline form. This is characteristic of the hexaacetate of *l*-inositol (4). The hexabenzoate was prepared by refluxing the crystalline *l*-inositol for five hours with benzoyl chloride and pyridine. The resulting mixture was made alkaline with 10% $NaHCO_3$ solution and extracted with three successive portions of ether. The combined ether extracts were washed with 5% HCl, 5% NaOH, and water, in that order. Evaporation of the ether yielded an oily brown residue, which was washed with absolute alcohol. The alcohol dissolved most of the impurities and left insoluble the white amorphous hexabenzoate. It was recrystallized twice from absolute alcohol, forming colorless prismatic needles which melted at 253° to 253.5° without decomposition. Tanret (4) 252°, Posternak (6) 253°. $[\alpha]_D^{25} = -67.7°$.

Anal.—Calcd. for $C_{48}H_{36}O_{12}$: C, 71.64; H, 4.51. Found: C, 71.90; H, 4.69.

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* Received June 22, 1951, from the School of Pharmacy, University of Wisconsin, Madison.
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‡ American Foundation for Pharmaceutical Education Fellow.

A Note on the Chemical Examination of the Fruits of *Terminalia balerica*, Roxb.*

By SURESH PRASAD SEN and JAGAT NARAYAN TAYAL

Terminalia balerica, Roxb., is indigenous to this country. It is commonly known as Bahera. It belongs to the *Combretaceae* family and grows throughout India. Many medicinal properties have been attributed to it in the Indian indigenous system of medicine. It is said to be astringent, laxative, and useful in scorpion sting, diseases of the liver and gastrointestinal tract (1). The kernel is believed to possess narcotic properties.

Kirtikar and Basu (2) have mentioned the presence of a yellow fixed oil in the drug, which is reported to be useful in rheumatism. The oil from its seeds has been examined for its constants by Shrikhande and Godbole (3). A preliminary report on the pharmacological actions of the crude fractions of the drug by Chakravarty and Tayal (4) suggested valuable therapeutic possibilities of the active components.

The drug was collected locally and identified by examination of its morphological characteristics. The fresh drug (entire fruit) was powdered in No. 60 powder and used in all experiments.

EXPERIMENTAL

The powdered drug was exhausted with boiling alcohol (95% v/v). The extracted portion was evaporated to dryness, yielding a dark greenish semisolid mass which was extracted several times with petroleum ether and separated into petroleum ether soluble and petroleum ether insoluble fractions.

Petroleum Ether Soluble Fraction.—This was filtered and freed from the solvent. A green fixed oil was obtained which gave the following constants on examination:

Acid value	21.89
Iodine value (pyridine bromide method)	75.82
Percentage of mixed acids	54.25
Iodine value of the mixed acids	71.5
Mean molecular weight of the mixed acids	280.0
Saponification value of the oil	185.85

Petroleum Ether Insoluble Fraction.—This fraction was treated with lead subacetate to precipitate tannins. The precipitate of lead tannate was termed fraction A, and the filtrate after removal of lead was designated fraction B.

Fraction A.—This was treated successively with cold and hot alcohols as well as acetic acid. The following fractions were separated.

(*a*) The alcoholic washings on evaporation yielded a *resinous residue.* This was found insoluble in ether and ethyl acetate, but soluble in chloroform. The chloroform extract on evaporation yielded a yellow precipitate with a pleasant odor.

(*b*) The acetic acid soluble fraction was examined and identified as a saponin. On hydrolysis it yielded glucose.

(*c*) The portion which remained insoluble in alcohol and acetic acid was pure tannin.

Fraction B.—Fraction B was treated with ethyl ether. The ethereal extract on evaporation left a yellow amorphous residue, which was readily soluble in water and gave tests for aldehydes. By repeated fractionation with ethyl ether, three different fractions (all glycosidal in nature) were separated. These fractions were amorphous and hygroscopic, with melting points ranging from 153° to 163° and varying in color from yellowish white to bright yellow.

Residual Alcoholic Portion.—This portion was soluble in water. The aqueous solution on concentration gave dark brown scales of resin yielding tests of a polyphenolic glycoside. The quantity of resin acids in the sample was estimated to be 5.89%.

* Received January 9, 1951, from the Central Drugs Laboratory, Calcutta, India.

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CORRECTION

In the article entitled, "The Colorimetric Determination of the Antihistaminic Drug N^1-*p*-Methoxy Benzyl-N^1-2-Pyridyl-N^1-Dimethyl Ethylene Diamine (Neohetramine)," by Milton Feldstein and Niels C. Klendshoj, [THIS JOURNAL, **40**, 370–372 (August, 1951)] make the following corrections: In the title, change the word "Pyridyl" to read "Pyrimidyl." In the second line of the right-hand column, page 370, change the word "pyridyl" to read "pyrimidyl."

Book Reviews

Pharmacopée Française. (Codex Medicamentarius Gallicus.) 7th ed. Edited under the direction of the Commission Permanente du Codex par L' Ordre National des Pharmaciens, Paris, 1949. xvi + 1227 pp. 16 x 24.3 cm.

The seventh edition of the French Pharmacopeia (French Codex 1949), which became available in 1951, is the first revision of this book of standards since 1937. This long delay in a period of rapid therapeutic change was caused by the 1939–1945 foreign occupation and post-liberation recovery.

A supplement issued in 1947 added 10 new medicinals to the Codex, and 11 more were added in 1949. Forty-seven new monographs are added in this edition. The new drugs are: acetylcholine chloride, aminoacetic acid, amphetamine sulfate, ascorbic acid, benzyl benzoate (pure and technical), benzylpenicillin sodium and potassium, butyl aminobenzoate, calciferol, cetyl alcohol, chloralose, choline chloride, cinchophen, D.D.T. (technical), desoxycorticosterone acetate, diacetyldiphenolisatin, dibucaine hydrochloride, *p*-dichlorobenzene, dihydrostreptomycin sulfate, diiodotyrosine, diphenylhydantoin and the sodium salt, dried citrated normal human plasma, estradiol, hexanestrol, hexobarbital, histamine hydrochloride, histidine hydrochloride, homatropine hydrobromide, hordenine sulfate, menadione, mercurescein sodium (merbromin), methylacetylcholine chloride, nicotinic amide, nikethamide, pentamethylenetetrazol, penthiobarbital sodium (thiopental sodium), pethidine hydrochloride, phenothiazine, stilbestrol, sulfadiazine, sulfaguanidine, sulfamerazine, sulfanilamide, sulfapyridine, sulfathiazole, testosterone acetate and propionate, thiamine hydrochloride, and thioarsphenamine. Standards are included for surgical sutures.

Aureomycin, terramycin, and other new antibiotics that are widely used in the United States are not found in this edition of the Codex. Many new drugs such as mepacrine (quinacrine), chloroquine, *p*-aminosalicylic acid, khellin, dicoumarol, heparin, and others, representing many new developments in therapeutic procedures, are included in a table which gives the common and scientific names of these drugs, but monographs for many of these important drugs are missing. The Codex of 1937 recognized the crystalline glycoside (digitoxin) from *D. purpurea*. This is still the only cardiotonic glycoside in the 1949 edition. No antihistaminic drugs are included.

A large number of crude drugs are retained in this edition although the beautiful pictures have been omitted. In many cases the drugs are used in galenicals but in some cases no use is indicated. The cumbersome size of the 1937 edition of the Codex has been cut down considerably and elimination of more crude drugs could have helped in that respect.

The monographs of many drugs contain information that is helpful to the pharmacist. The pharmaceutical use of drugs is indicated. The classification of drugs according to the official poison tables is given. Penthiobarbital sodium and phenobarbital are classified as: DANGEROUS—Table C. Codeine as: TOXIC—Table A. Opium and Cocaine as: STUPEFIANT (NARCOTIC)—Table B. These tables were included in a separate volume of the 1937 edition, along with legal regulations, tabulated data on drugs and drug products that had appeared in the Codex since 1818, and even sample labels for veterinary products, solutions for injection, and dangerous drugs were given. Most of this information is omitted from the 1949 edition, including the poison tables.

Drugs and preparations that are intended for veterinary use have this indicated in the monographs. A six-page section is devoted to phytopharmaceutic preparations or plant pesticides and sprays.

Incompatibilities are given for some drugs. Under sodium salicylate incompatibilities are given as: acid solutions; antipyrine (a mixture of sodium salicylate and antipyrine becomes syrupy after several hours and gives an alkaline reaction).

Dosage tables are given for adults and for children. The table for adults gives the mode of administration, the single usual and maximum doses, and the daily usual and maximum doses. The table for children gives the mode of administration, the daily dose for infants up to thirty months and for children from thirty months to fifteen years.

A monograph on aerosol medicaments is included in this new edititon of the French Codex. An interesting innovation is the identification of the individual responsible for each monograph by placing his initials at the end. It is pleasant to contemplate the artistic French in their scientific treatise. The first monograph in this edition, as in the preceding edition of the French Codex, is on absinthe. The description of the plant ends with the statement that the leaves and tops have a very strong and special odor, an aromatic bitter flavor.

The 1949 French Codex is a worthy and useful addition to the new pharmacopeias. The newer drugs that are mentioned but not described in this edition will undoubtedly appear in interim supplements.

Marine Products of Commerce. 2nd ed. By DONALD K. TRESSLER and JAMES McW. LEMON. Reinhold Publishing Corporation, New York, 1951. xiii + 782 pp. Illustrated. 16 x 23.8 cm. Price $18.

This book, written by two authorities on foods and fisheries with the aid of 14 experts in specialized lines, is a complete treatise dealing with commercially important products of the sea. Descriptive information covers products of the oceans, the technique of obtaining them, and their subsequent treatment in processing.

Essential information not readily available elsewhere on manufacturing processes; process machinery; the equipment used for the transportation of fresh and frozen fishery products; methods of rapid freezing; and important technological developments in feed for animals extracted from seaweed, moss, and alginates are presented.

Since publication of the original edition 27 years ago, progress in the technology of marine products has been so rapid that the new edition was entirely rewritten and expanded. It presents considerations of the world's agar industry; the first attempted statistical survey of the world's fisheries and shellfisheries and their many products; and serves as a source of information on the technology of fish glue manufacture. It presents what appears to be an excellent outline of useful information on oceanography, chemical technology, marine botany and biology, conchology, ichthyology, the marine fisheries, fishery technology, statistics, nutrition, fishery by-products, shell-fisheries, whaling, sealing, sponge fishing, and gemology.

The book is documented with references to many specialized treatises on the several subjects covered. Its usefulness is enhanced by an excellent index.

Maize in the Great Herbals. By JOHN J. FINAN. The Chronica Botanica Co., Waltham, Mass.; Stechert-Hafner, Inc., New York, 1951. xiv + 43 pp. Illustrated. 17.5 x 25.5 cm. Price $3.

This newest product of the Chronica Botanica Company follows the line that has become characteristic of its publication policy. This policy includes the illumination of scientific problems and/or development by tracing their progress in art and philosophy, as well as in science proper.

How this tendency has expressed itself in the booklet under consideration, and how the correlation of botanical knowledge and research with the helpful endeavor of an art historian and experts on medieval Latin and Spanish history has worked in this scholarly master thesis of a Pioneer Hi-Bred Corn Company Fellow in Washington University has been delightfully told in the foreword by Edgar Anderson, the well-known botanist and geneticist at Washington University and the Missouri Botanical Garden. It is understood that this making of "*unum ex pluribus*," leading not only to an effective and reliable story but even to new conclusions, called for tact, taste, and careful consideration by the author, J. J. Finan, and the teachers of the various faculties who helped and guided him.

The new conclusions referred to have resulted in the certainty "that two general types of maize are discussed in the herbals," one similar to the typical flints of eastern North America and believed by most of the herbalists to have been brought into Europe from Asia, and another much like the present-day corn of the Caribbean area and very likely introduced into Europe by the early Hispanic explorers.

The use of herbal illustrations as a means of research rather than for decorative purposes being one of the most remarkable features of this essay, it is but appropriate that a brief but pertinent explanation of "the woodcuts of the great herbals" (Appendix I) is added. The essay appeared first in the *Annals of the Missouri Botanical Garden*, **35**, 149 (1948). This hand-bound, limited, and very attractive edition was designed by the meritorious editor of *Chronica Botanica*, Dr. Frans Verdoorn.

It might be of interest to those especially interested in pharmaco-botany that similar attempts at following up the history of medicinal plants by methodically examining the herbals and books on drugs from antiquity up to the late eighteenth century have been going on for quite a while. As a matter of fact, the herbals themselves refer again and again to the concepts of earlier authors.

A study by Hedwig Schwarz resembling that of Finan in the use of plant illustrations as a means of research (and dealing with *Chelidonium majus* and *Paeonia peregrina* Mill.—*Paeonia officinalis* L.) was published in 1931 by the *Gesellschaft für Geschichte der Pharmazie*. From 1930 to 1937 there were presented at the University of Basel a number of Doctor's theses as contributions to the history of official drugs ("*Beitrgäge zur Geschichte der Offizinellen Drogen*"), prepared under the guidance of Professor Heinrich Zörnig (and partly with the advice of Professor J. A. Häfliger), which had the examination of the herbals and books on herbs through the ages as their basis.

In most recent time pharmaceutico-botanical research, including the historical tracing of origins, has been paid much attention in the German pharmaceutical press. Permanent rubrics have been devoted to it in *Die Pharmazie*, founded in 1946, and in the *Arzneimittel-Forschung*, founded in 1951.—GEORGE URDANG.

The Enzymes—Chemistry and Mechanism of Action. Vol. I, Part 2. Edited by JAMES B. SUMNER and KARL MYRBÄCK. Academic Press, Inc., New York, 1951. x + 637 pp. 16 x 23.5 cm. Price $12.80.

This book continues with the plan of Volume I, Part 1, by taking up specific groups of enzymes, the first part of Volume I having dealt largely with general properties of enzymes. This book covers 24 different types, including such well-known groups as proteolytic enzymes, urease, phosphorylases, and some of the less familiar ones as thiaminase, cellulases, penicillinase and histidase. There is also a more general chapter entitled "Enzymes as Essential Components of Bacterial and Animal Toxins," by E. A. Zeller.

The high quality found in Volume I, Part 1, is maintained in this book, with the chapters being written by men well qualified by experience in their subjects. All sections are thoroughly referenced. The pages are numbered consecutively with Volume I, Part 1, and the index includes both parts of Volume I.

The practice of introducing each chapter by giving the reader some insight into the occurrence, historical development, and significance of each group of enzymes is commendable. Many tables are used to summarize pertinent data cited, and many graphs are included to depict *p*H effects, reaction kinetics, and inhibitor effects. Of special value is the inclusion by the authors of methods of assay and measurement of activities along with some criticism, usually of the methods.

This book is a significant contribution to the literature on enzymes, and it is hoped that future volumes will meet the high standards set by Volume I.

Preparation of Organic Intermediates. By DAVID ALLEN SHIRLEY. John Wiley and Sons, Inc., New York, 1951. x + 328 pp. 15.5 x 23.5 cm. Price $6.

There are many useful organic compounds employed as intermediates in synthesizing other compounds which are either not available commercially or are inordinately expensive. Directions for the preparation of many of these chemicals are included in *Organic Syntheses*, but some are not described therein.

This book is designed to provide the synthetic organic chemist with specific preparative directions for approximately 500 organic compounds for which methods are not included in *Organic Syntheses*. In addition to this criterion for the inclusion of a method of preparation in this book, the structure of the compound must be relatively simple and must contain reactive functional groups that make it useful as an intermediate.

The author has been careful to include only synthetic methods involving the use of starting material either readily available commercially or readily prepared from directions published in *Organic Syntheses* or in another place in the present volume. Many compounds of special interest to pharmaceutical chemists engaged in synthetic medicinal chemistry research are included. Methods for so many compounds of this type may have been influenced by the author's interest in research on the chemotherapy of tuberculosis and in heterocyclic compounds.

The book is provided with a molecular formula index, a type of reaction index, and a general index, all three of which contribute to the usefulness of the book. Reference to the original source of each method of preparation is, of course, included in each monograph.

Organic Reactions. Vol. VI. ROGER ADAMS, Editor-in-Chief. John Wiley and Sons, Inc., New York, 1951. viii + 517 pp. 15.5 x 23.5 cm. Price $8.

Started in 1942, this represents the sixth volume of a series designed to provide a compilation of all the important synthetic chemical reactions. As in the previous volumes of the series, each chapter has been written by authors who have had actual experience with the reactions described and reviewed.

The design of each chapter in this volume follows that established in the first number of the series. In general, a discussion of the method, modification, examples of applicability, special precautions, a detailed description of procedure, expected yields, and other pertinent data are included. Tables for each chapter have been prepared in which compounds synthesized by or subjected to the reaction under discussion are listed. The contents of Volume VI include The Stobbe Condensation; The Preparation of 3,4-Dihydroisoquinolines by the Bischler-Napieralski Reaction; The Pictet-Spengler Synthesis of Tetrahydroisoquinolines and Related Compounds; The Synthesis of Isoquinolines by the Pomeranz-Fritsch Reaction; the Oppenauer Oxidation; The Synthesis of Phosphonic and Phosphinic Acids; The Halogen-Metal Interconversion Reaction with Organolithium Compounds; The Preparation of Thiazoles; The Preparation of Thiophenes and Tetrahydrothiophenes; and Reductions by Lithium Aluminum Hydride.

Volume VI of *Organic Reactions* constitutes a contribution which, it is believed, will be as enthusiastically received by organic chemists as were its predecessors. The editor and the publishers are to be congratulated on the vision and judgment exercised in making this series of books available.

BOOKS RECEIVED

Methods in Medical Research. Vol. 4. MAURICE B. VISSCHER, Editor-in-Chief. Year Book Publishers, Inc., Chicago, 1951. xiv + 306 pp. Illustrated. 14.5 x 22.3 cm. Price $7.

Six-Membered Heterocyclic Nitrogen Compounds with Four Condensed Rings. By C. F. H. ALLEN. Interscience Publishers, Inc., New York, 1951. xiii + 345 pp. 16 x 23.8 cm. Price $10.

Reagent Chemicals. American Chemical Society Specifications, 1950. American Chemical Society, Washington, D. C., 1951. xiii + 400 pp. 16 x 24.2 cm. Price $5.

Substances Naturelles de Synthese. Vol. II. By LEON VELLUZ. Masson & Cie, Paris, 1951. 138 pp. 16 x 23.3 cm.

The Pharmacologic Principles of Medical Practice. 2nd ed. By JOHN C. KRANTZ, Jr. and C. JELLEFF CARR. The Williams & Wilkins Co., Baltimore, 1951. xvii + 1116 pp. Illustrated. 16 x 23.5 cm. Price $10.

Science French Course. By C. W. PAGET MOFFATT. Revised by NOEL CORCORAN. Chemical Publishing Co., Inc., Brooklyn, N. Y., 1951. viii + 332 pp. 12 x 19 cm. Price $4.75.

An Introduction to the Chemistry of the Silicones. 2nd ed. By EUGENE G. ROCHOW. John Wiley and Sons, Inc., New York, 1951. xiv + 213 pp. Illustrated. 15 x 23.5 cm. Price $5.

Organic Reactions. Vol. VI. ROGER ADAMS, Editor-in-Chief. John Wiley and Sons, Inc., New York, 1951. viii + 517 pp. 15.5 x 23.5 cm. Price $8.

Pharmacopoeia Japonica VI. Japanese Pharmaceutical Association, Tokyo, Japan, 1951. 756 pp. 15 x 21.5 cm.

Biology for Pharmaceutical Students and Others. 2nd ed. By S. MANGHAM and A. R. HOCKLEY. Baillière, Tindall and Cox, London, 1951. viii + 634 pp. Illustrated. 14.5 x 23.5 cm. Price $6.50.

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